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The Reaction of Oxidizing Agents with Wool

5. THE OXIDATION PRODUCTS OF THE DISULPHIDE BOND AND THE FORMATION OF A SULPHONAMIDE IN THE PEPTIDE CHAIN

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It has long been recognized that the reaction between wool keratin and oxidizing agents is extremely complex (see Stoves, 1946). Although most oxidizing agents act on the disulphide bond, other groups are also oxidized and considerable general degradation is found. As the disruption of the disulphide bond alone causes a great reduction in the strength of the fibres, it is difficult to detect limited main chain breakdown. In the work described below, one of the reagents used was peracetic acid which acts specifically on the disulphide bonds and tryptophan side groups and undesirable reactions are eliminated (Alexander, Hudson & Fox, 1950).

It has hitherto been customary to interpret the reaction of the combined cystine in keratin in the same way as the reactions of cystine or similar disulphides. Cystine is oxidized by perbenzoic acid in chloroform to a disulphoxide which is readily converted to the corresponding sulphonic and sulphinic acids by 95 % methanol and hydrochloric acid (Lavine, Toennis & Wagner, 1934).

$$\mathrm{RSSR} \rightarrow \mathrm{R.\overset{\overset{O}{\|}}{S}.\overset{\overset{O}{\|}}{S}.R} \rightarrow \mathrm{RSO_3H + RSO_2H}$$

In this reaction an intermediate R.SO.SO.R was isolated which was found to be relatively stable in dilute acids (Lavine, 1936), but which reacts with ammonia to form a complex mixture including cystine and the sulphinic acid corresponding to cysteine. Speakman & Elliott (1940) showed that dibenzyl disulphide is oxidized by sulphuryl chloride to the sulphur chloride in non-aqueous media, and for this reason Speakman (1945) has postulated the formation of sulphur chlorides to be the primary reaction between wool and chlorine. Harris & Smith (1937) assumed that cysteic acid is the main product in the reaction with hydrogen peroxide, although some evidence for the formation of an intermediate sulphoxide was advanced. They also deduced that bond fission occurs only when one of the sulphur atoms is converted to cysteic acid and that both sulphur atoms are not oxidized simultaneously. Consden, Gordon & Martin (1946) found cysteic acid in hydrolysates of wool treated with bromine. Consden & Gordon (1950) showed the presence of intermediary oxidation products, possibly the sulphoxide and the sulphinic acid, in wool oxidized with hydrogen peroxide.

Alexander *et al.* (1950) found that the combined cystine in wool could be divided into two fractions which are recognized by their behaviour towards oxidizing agents. Chlorine in acid solution and peracetic acid oxidize all the cystine, whereas potassium permanganate in acid solution and sodium hypochlorite oxidize only 25 %. The results presented in the present paper show that the nature of the products formed depends upon the cystine fraction reacting. It is shown that the reactions of the cystine in wool differ considerably from the corresponding reaction of free cystine, and this is not surprising in view of the stereochemical restrictions which must exist in the three-dimensional network of cross-linked polypeptide chains. In the reactions with peracetic acid and chlorine at pH 2, it was found that the disulphide bond was broken and that subsequent hydrolysis gave cysteic acid quantitatively. Cation-exchange experiments showed that free sulphonic acid groups were not present in the wool fibres after oxidation so that an intermediate compound must be formed. Further, after treatment of the oxidized fibres with dilute alkali a product was obtained which did not yield cysteic acid on hydrolysis by the normal method. Acidic compounds were extracted from such hydrolysates, which on drastic hydrolysis gave cysteic acid. Evidence is presented which indicates that the oxidized sulphur is present as a cyclic sulphocarboxylic acid imide, which gives rise to a sulphonamide on treatment with alkali.

EXPERIMENTAL

The wool fabric used throughout this work was loosely knitted from a high quality botany yarn and had received no prior treatment other than mild scouring with NH_3 to remove wool grease and dirt. It was further purified by thorough extraction with ether and ethanol.

Oxidations. These were performed as described by Alexander *et al.* (1950).

Cystine determinations. Wool samples were hydrolysed with 5N-HCl at 120° for 5 hr. in sealed tubes. The cystine content of the hydrolysate was determined colorimetrically with phosphotungstic acid (Shinohara, 1935).

Cysteic acid determinations. Wool hydrolysates were chromatographed on Whatman no. 4 paper in phenol-0·3N-NH_3 (Consden, Gordon & Martin, 1944). Cysteic acid was estimated on these chromatograms by the spot dilution method (Polson, Mosley & Wyckoff, 1947).

Two-dimensional paper chromatography. The chromatogram was run in one direction with phenol (8-hydroxyquinoline addition) and then at right angles with *s*-collidine.

Total sulphur. The wool S was oxidized to sulphate by a modification of the method of Kahane & Kahane (Jones, 1943). Frequent control analyses gave an average value of 3·50% S for whole wool. The reproducibility of the results was better than ±3%.

Cation exchange. Oxidized wool samples (1 g.) were shaken successively with six lots of 30 ml. of N-NH_4Cl solution. They were washed with distilled water till free of Cl^-. The samples were then shaken successively with six lots of 30 ml. of N-KCl. All KCl solutions were bulked after shaking and tested for NH_4^+ qualitatively with Nessler's solution and quantitatively by boiling with NaOH and absorbing the NH_3 in standard acid (see Kressman & Kitchener, 1949). Alternatively, oxidized wool samples which had been equilibrated by successive shakings with distilled water were shaken with six lots of 30 ml. of N-KCl and the liquors bulked and titrated against standard alkali.

Acid-binding capacity of wool. Wool samples (1 g.) were equilibrated to pH 6 by successive shakings with N-KCl. The samples were freed from excess water by squeezing and shaken for 30 min. in a flask shaker with 30 ml. of 0·05N-HCl containing N-KCl. Samples of the solution were then back titrated with standard alkali.

Load-extension of fibres. Tensile measurements on fibres were carried out as described by Speakman (1947).

RESULTS

The difference in products on acid and alkaline oxidation

Chlorination at pH 2, 4 *and* 8. The yields of cysteic acid obtained in the hydrolysates of wool which had been chlorinated at pH 2, 4 and 8 are recorded in Table 1. All the cystine which reacted was converted quantitatively to the sulphonic acid when 100 g. of wool were reacted with up to 20 g. of chlorine; but with greater quantities of chlorine, loss of cysteic acid occurred. This is supported by analyses for total sulphur, as the sum of the disulphide and cysteic acid sulphur remains constant for the various treatments.

The relatively large amount of non-disulphide sulphur in wool has been investigated by Cuthbertson & Phillips (1945) who conclude that all the sulphur in virgin wool can be accounted for as disulphide sulphur and methionine. At pH 8, oxidation did not give cysteic acid quantitatively, as the solution contained an appreciable concentration of hypochlorite ions. At that pH the reaction proceeds partly with hypochlorous acid and partly with hypochlorite ions (Alexander *et al.* 1950) and a quantitative conversion to cysteic acid cannot be expected.

Table 1. *Disulphide sulphur, cysteic acid and total sulphur analyses of wool treated with various concentrations of chlorine at* pH 2, 4, 8 *at* 25°

		g./100 g. wool		
pH	Chlorine reduced (g./100 g. of wool)	Disulphide sulphur	Cysteic acid sulphur	Total sulphur
2·0	Nil	2·66	—	3·50
	5	2·05	0·6	3·57
	10	1·57	1·3	3·35
	20	0·72	1·9	3·40
	50	0·27	1·4	2·50
4·0	20	0·98	1·7	3·54
	50	0·16	1·7	2·80
8·0	20	2·17	0·1	3·33
	50	1·40	0·4	2·69

Chlorine solutions at pH 2–8, though reacting preferentially with the cystine and tyrosine in wool (Alexander & Gough, 1951), do not confine their attack to these amino-acids. In so far as can be ascertained from paper chromatograms of chlorinated wools there is no difference between these and untreated wool except for the disappearance of tyrosine and cystine and the appearance of cysteic acid. It appears, therefore, as if a general attack on all the other amino-acids occurs and it seems likely that this proceeds via the terminal amino-acids. Tryptophan may be oxidized preferentially together with tyrosine and cystine, but since it does form a significant part of the wool it was not determined.

Chlorination at pH 10. Chlorine in solution at pH 10 consists almost entirely of hypochlorite ions which are found to yield a different reaction product. No cysteic acid was detected in the hydrolysates of wool treated with chlorine at pH 10,* and two-dimensional chromatograms revealed lanthionine. Cuthbertson & Phillips (1945) have shown this amino-acid, which was first isolated by Horn, Jones & Ringel (1941) from alkali-treated wool, to be present in wool boiled with pH 10 buffer. Unlike cysteic acid, the lanthionine could not be estimated quantitatively, but its presence was confirmed by the technique of Dent (1947), using hydrogen peroxide.

The value for the lanthionine sulphur (Table 2) was not obtained directly, but was calculated on the assumption, made by Cuthbertson & Phillips (1945), for evaluating the lanthionine in alkali-treated wool, that all the sulphur can be accounted for as lanthionine, disulphide and non-disulphide sulphur

* The reaction mixture was found to contain a small amount of cysteic acid at the end of the reaction.

(0·88 %) of untreated wool. The slight loss in total sulphur compared with that of untreated wool is in agreement with the formation of lanthionine in the alkaline degradation of wool (Cuthbertson & Phillips, 1945). The eliminated sulphur which is seen to correspond to approximately one-half of the disulphide sulphur lost could not, however, be detected as sulphate in the reaction liquor. Cuthbertson & Phillips (1945) also were not able to isolate part of the sulphur-containing reaction products formed during the alkaline degradation of wool.

Table 2. *Analyses of disulphide sulphur and total sulphur in wool treated with various amounts of chlorine at* pH 10 *at* 25°

Chlorine reduced (g./100 g. of wool)	g./100 g. wool		
	Disulphide sulphur	Lanthionine sulphur*	Total sulphur
5	2·08	0·34	3·31
20†	1·86	0·58	3·32
50	1·86	0·48	3·22

* Calculated as the difference between the total sulphur and the sum of the cysteic acid and original non-disulphide sulphur (0·88 %).

† Reaction performed at 80°.

The formation of cross links in wool fibres treated with sodium hypochlorite at pH 10 is confirmed by the load-extension data given in Table 3. The work required to stretch the fibre to a constant extension was greatly reduced by chlorination at pH 2. After chlorination at pH 10, however, the reduction in work (R.W.) was small, suggesting that new cross links (i.e. lanthionine) were formed after the oxidation of the disulphide bonds. Furthermore, a peracetic acid treatment sufficient to oxidize all the disulphide bonds does not render wool which had been chlorinated at pH 10 soluble in dilute ammonia (cf. Fig. 1).

Table 3. *The reduction in work* (R.W.) *obtained on stretching the fibres to a constant extension, after treatment with chlorine solutions at* pH 2 *and* 10 *at room temperature*

pH	Chlorine reduced (g./100 g. of wool)	Percentage cystine oxidized (percentage of that originally present)	R.W. at pH 7
2·0	4	16·5	21
	6	25·5	35
10·0	4	20·0	3·3
	6	22·2	4·6
	8	—	10·4
	10	22·4	14·4

Effect of acid permanganate. In the treatments with acid permanganate solutions, analyses for cystine and cysteic acid showed that approximately one-half the cystine oxidized was converted to sulphonic acid (Table 4). Most of the sulphur which is removed from the wool could be recovered from the reaction mixture as sulphate (e.g. 0·18 % sulphur was found as sulphate after a potassium permanganate treatment, 25 g./100 g. wool).

Table 4. *Analyses for disulphide, cysteic acid and total sulphur in wool treated at* 25° *with* $KMnO_4$ *in* 0·5N-H_2SO_4

(Total sulphur of untreated wool = 3·5 %.)

$KMnO_4$ reduced (g./100 g. of wool)	g./100 g. wool		
	Disulphide sulphur	Cysteic acid sulphur	Total sulphur
12·5	2·10	0·3	3·22
25·0	1·87	0·3	3·20

Alkaline permanganate treatment. The cystine in wool is only attacked by alkaline permanganate solutions when a large excess of the oxidizing agent is used, causing severe degradation (Alexander *et al.* 1950). The high sulphur content of the treated wool suggests that the reaction is completely non-specific for cystine and it appears that other residues are preferentially attacked. Thus, a sample which had received treatment with 25 g. permanganate/100 g. wool had a sulphur content of 3·88 % compared with a normal value of 3·50 % showing that other residues had reacted and passed into solution leaving the cystine mainly unattacked. No cysteic acid was observed on acid hydrolysis of the treated wools, but two-dimensional paper chromatography revealed some lanthionine.

Table 5. *Analyses for disulphide, cysteic acid and total sulphur on wool treated with* 0·25N-*peracetic acid* (270 % *on wt. of wool*)

Time of reaction in 0·25N-peracetic acid	g./100 g. wool		
	Disulphide sulphur	Cysteic acid sulphur	Total sulphur
5 min.	2·10	0·5	3·60
30 min.	1·44	1·2	3·50
1 hr.	0·66	2·0	3·46
25 hr.	0·27	2·3	3·56

Peracetic acid. The hydrolysates of wool samples which had been treated with peracetic acid were estimated for cysteic acid. Within the experimental error of the method quantitative conversion of the combined cystine to the sulphonic acid was observed (Table 5). All the sulphur is accounted for as cysteic acid even when 90 % of the cystine has reacted in contrast to chlorination in an acidic medium. The reason for this is that the peracetic acid, unlike chlorine, reacts specifically with the cystine and no side reactions involving oxidized cystine take place.

After complete oxidation of the cystine with peracetic acid 90 % of the wool became soluble in dilute alkali. Fig. 1 shows the relation between the solubility of wool in cold 3N-ammonia and the proportion of cystine oxidized by peracetic acid and chlorine at pH 2. The greater solubility of chlorinated wool when less than 80 % of the cystine was oxidized also indicates the non-specific nature of the reaction with chlorine.

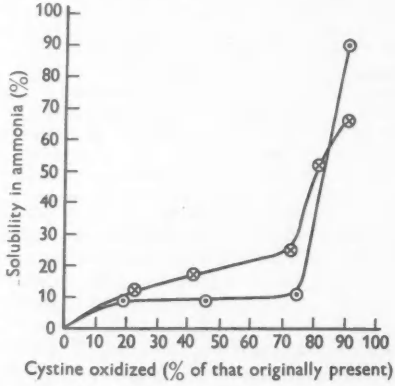


Fig. 1. Relationship between solubility of wool in 3N-ammonia and the proportion of cystine oxidized by chlorine solutions at pH 2, —⊗—; and peracetic acid, —⊙—.

After chlorination in acid the tensile strength of single fibres was greatly reduced (Table 3). Load-extension data on fibres which had been oxidized with 0·25N-peracetic acid showed that a similar reduction in work (R.W.) occurs. This reduction increased with the extent of disulphide bond oxidation (Table 6).

It was also observed that the difference in R.W. at pH 1 and 7 found with untreated fibres disappeared on extensive oxidation of the disulphide bond. According to Speakman (1947), the increased tensile strength of untreated fibres at pH 7 is due to the formation of salt linkages which are broken at pH 1. Sookne & Harris (1937), however, attributed the difference in strength to increased swelling in acid solution. The above results support this latter view, as Harris, Mizell & Fourt (1942) showed that swelling of the fibre increased with increasing disulphide bond breakdown. It appears, therefore, that swelling is the operative factor responsible for the difference in elasticity of wool fibres in acid and neutral solutions, the breaking of salt linkages being only of secondary importance.

Table 6. *The reduction in work* (R.W.) *on stretching the fibres to a constant extension, after treatment with* 0·25N-*peracetic acid at room temperature*

Time of treatment in 0·25N-peracetic acid (min.)	Cystine oxidized (percentage of that originally present)	R.W. at pH 1	R.W. at pH 7
—	0	38	2
5	19·8	48	28
30	46·3	70	67
60	71·1	82	84
120	75	93	97

It is clear that oxidation with peracetic acid or chlorine solution at pH 2 disrupts the disulphide bond in such a way as to yield cysteic acid on subsequent acid hydrolysis. In order to determine whether combined cysteic acid formed within the fibre or only resulted on acid hydrolysis, experiments were made in order to exchange the H^+ or NH_4^+ ions initially absorbed on any free sulphonic acid groups within the fibre by potassium ions using the procedure already described. No exchange could be detected in any of the experiments performed, showing that free cysteic acid does not exist within the fibres.

The nature of the oxidized disulphide bond

Ammonia extraction of wool oxidized in acid solution. Wool samples which had been oxidized with peracetic acid or chlorine at pH 2 were allowed to stand overnight in cold 3N-ammonia. On hydrolysis with 15 % HCl at 125° for 5 hr. very little cysteic acid could be detected, although the sulphur content of the wool had not decreased appreciably (Table 7).

Table 7. *Analyses for cysteic acid sulphur and total sulphur contents of wool oxidized with peracetic acid and chlorine at* pH 2, *before and after alkaline extraction with* 3N-NH_3

Treatment	g. S/100 g. wool: Cysteic acid sulphur, Before extraction	Cysteic acid sulphur, After extraction	Total sulphur, Before extraction	Total sulphur, After extraction
Cl_2 (5 g./100 g. wool)	0·6	Nil	3·57	3·40
Cl_2 (10 g./100 g. wool)	1·3	0·1	3·35	3·40
Cl_2 (20 g./100 g. wool)	1·9	0·2	3·40	3·12
5 min. in 0·25N-peracetic acid	0·5	0·1	3·50	3·35
30 min. in 0·25N-peracetic acid	1·2	0·3	3·50	3·16
1 hr. in 0·25N-peracetic acid	2·0	0·2	3·46	2·97

The loss in weight on extraction with 3N-ammonia (see Fig. 1) was small until more than 80% of the cystine had been oxidized.

Similar results were obtained when 0·1N-NaOH was used instead of 3N-NH_3. Wool, for example, which had been treated for 1 hr. with excess peracetic acid and subsequently shaken with 0·1N-NaOH for 0·5 hr. had a total S content of 2·95%, although the hydrolysate contained a negligible quantity of cysteic acid. Experiments with peptides containing cysteic acid showed that such treatment with dilute alkali did not produce a loss of cysteic acid. The soluble fraction obtained when wool treated at pH 2 with 20 g. chlorine/100 g. of wool was extracted with dilute NH_3 solution, consisted of material with a total S content of 3·37%. Hence cysteic acid is not preferentially extracted, as untreated wool contains about 3·50% sulphur. It was found that the peptide hydrolysate contained 2·2% cysteic acid sulphur in contrast to the low cysteic acid content of the larger insoluble fraction.

Acid-combining capacity of alkaline-extracted oxidized wool. The fibres produced by oxidation in acid followed by extraction with alkali showed an increase in acid-combining capacity. Except for the 18 hr. treatment (see Table 8) the wool was shaken for the times indicated in each case. The results show that the product of this dual treatment contains approximately 45% more acidic groups than the untreated fibres. No increase in acid-binding capacity was observed with untreated fibres or fibres which had been oxidized with peracetic acid or chlorine in acid, and not treated further with alkali (see also Lemin & Vickerstaff, 1946).

Table 8. *Maximum acid-combining capacity of wool oxidized for* 1 *hr. with* 0·25N-*peracetic acid and extracted with* 3N-NH_3 *for various times*

Time of treatment with NH_3	Maximum acid combined* (g.equiv./1000 g. wool)
Nil	0·80
3 min.	0·98
5 min.	0·95
15 min.	1·11
18 hr.	1·15
30 min. (0·1N-NaOH)	1·04

* The value for untreated wool was 0·82.

That the increased titration is not due to the liberation of free carboxyl groups resulting from peptide chain breakdown during the mild alkaline treatment, is shown by load-extension data obtained with alkali-extracted fibres. No difference was found in tensile strength with fibres which had been treated with 3% chlorine at pH 2 before and after alkali extraction.

Hydrolysis of the product formed by oxidation in acid followed by alkali extraction. The unknown product formed after oxidation in acid and treatment with dilute alkali was found to be very stable to hydrolysis with acid and to cause the liberation of free acidic groups during its formation. That the unknown is not a lower oxidation product of cystine was shown by treating the hydrolysate with potassium permanganate solution when very little cysteic acid was produced. It was concluded that the oxidized sulphur combines with the imino nitrogen in the main polypeptide chain and gives a sulphonamide on mild alkaline hydrolysis. The latter are known to be very stable to acid and alkaline hydrolysis. In attempts to hydrolyse the suspected sulphonamides it was found that hydrolysis with concentrated hydrochloric acid for relatively short periods or prolonged hydrolysis with 25% (w/v) hydrochloric acid gave maximum yields of cysteic acid. Thus, oxidation of wool with 20% chlorine at pH 2 gave 1·9% of cysteic acid sulphur on hydrolysis with 15% (w/v) hydrochloric acid for 5 hr. at 125°. After treating the oxidized wool with dilute ammonia only 0·2% cysteic acid sulphur was obtained by hydrolysing under the same conditions, whereas hydrolysis with 25% (w/v) hydrochloric acid at 125° for 4 days gave a value of 1·0% cysteic acid sulphur, and hydrolysis with 32% (w/v) hydrochloric acid at 125° for 5 hr. resulted in a 0·8% yield. All the hydrolyses were carried out in sealed tubes. Control experiments showed that under the conditions employed, cystine was not oxidized and the destruction of cysteic acid was negligible.

Attempted separation of sulphonamides. The hydrolysate of a wool sample (15% (w/v) hydrochloric acid for 5 hr. at 125°) which had been oxidized with peracetic acid or chlorine at pH 2 to different extents and then extracted with 3N-ammonia for 20 hr. was desalted (Consden, Gordon & Martin, 1947) and the pH adjusted to 3–4. The solution was then applied to a column of Deacidite *B* (see Consden, Gordon & Martin (1948) who used Amberlite IR-4) to separate the acidic amino-acids from the remainder which passed through. The former were eluted from the column with N-hydrochloric acid. The small amount of cysteic acid originally present in the hydrolysate was not eluted by this procedure. The acidic fraction was again desalted, adjusted to pH 3–4, applied to the Deacidite column and eluted with water (Consden, Gordon & Martin, 1949). Several fractions were collected and shown by one-dimensional paper chromatography to contain aspartic and glutamic acids only. When these fractions were hydrolysed with concentrated hydrochloric acid at 125° for 5 hr. and again chromatographed in phenol-ammonia, every amino-acid including cysteic acid appeared.

Partial oxidation products

In addition to the main reaction giving a product which yields cysteic acid on acid hydrolysis, some evidence for the formation of an intermediate oxidation product of cystine after chlorination in acid

solution was obtained. The cystine content increased after alkali treatment (Table 9). The cystine produced by alkali treatment was probably derived by

Table 9. *The cystine content of wool oxidized by chlorine solution at* pH 2, *before and after extraction with* 3N-NH_3

Chlorine reduced (g./100 g. of wool)	Cystine content (%) Before extraction	After extraction
Nil	10·00	9·65
13	5·32	6·60
35	2·02	2·95
50	0·39	2·20

hydrolysis from cystine disulphoxide or some other incomplete oxidation product, e.g.

$$3RSO.SOR + 4NaOH \rightarrow RS.SR + 4RSO_2Na + 2H_2O$$

(Lavine, 1936).

After reducing with a mixture of 5N-potassium iodide and 5N-hydrochloric acid (see Lavine, 1936) the cystine content of a sample of wool which had been oxidized with 50% chlorine at pH 2 rose from 0·39 to 1·46%. Reduction under these conditions occurs only with oxidation products below the sulphonic level. No evidence for the presence of such products in wool oxidized with peracetic acid was found.

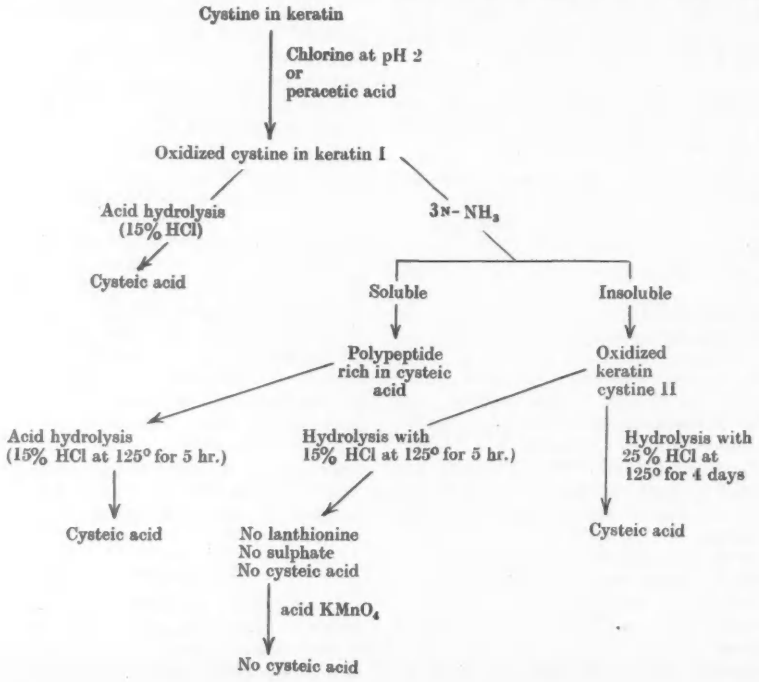
DISCUSSION

The analytical results show that the combined cystine in wool keratin may be oxidized under different conditions to give different products depending on the particular fraction which is reacting. Thus, oxidizing agents capable of reacting with all the cystine in wool gave products which were converted quantitatively to cysteic acid on subsequent acid hydrolysis. On the other hand, acid potassium permanganate which oxidized only 25% of the cystine, gave a mixture of cysteic acid and sulphate. The conversion of both sulphur atoms to cysteic acid may be prevented by steric factors so that the permanganate reacts with part of the side chains in addition, thus eliminating one sulphur atom/disulphide bond as sulphate.

Oxidation in alkaline solution

In alkaline solution, however, the 25% fraction capable of reacting is converted to lanthionine by permanganate and hypochlorite. The state of combination of the ejected sulphur atom is unknown (see Cuthbertson & Phillips, 1945).

Lanthonine has been produced by the action at 100° of alkaline buffers on wool (Cuthbertson & Phillips, 1945), although only 50% of the total



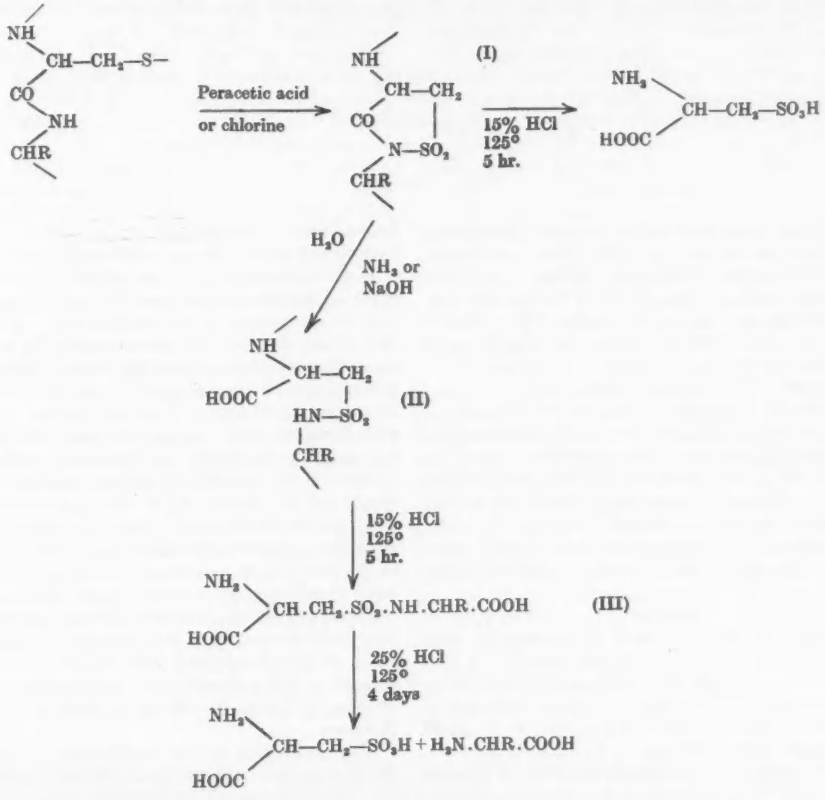
cystine reacted in this way. In the present experiments, however, the conversion to lanthionine was rapid in the cold, so that the hypochlorite ions must enter into the reaction. Cuthbertson & Phillips (1945) suppose their 50 % fraction to be linked in the molecule in such a way that the residues formed by the fission of the disulphide bond remain in close proximity and readily condense to form the monosulphide bond. Alexander & Earland (1950*b*) showed that only a small proportion of the sulphur is associated with the long polypeptide chains, the greater proportion residing in much shorter chains. It is suggested that the latter act as a cement between the micelles formed from the high molecular weight fraction. It is tempting to correlate the difference in reactivity of cystine with oxidizing agents, with the two protein components postulated in this model. There is no evidence, however, to enable the cystine fractions to be identified with either the high or low molecular weight polypeptides.

Formation of new cross-links by hypochlorite is supported by observations (Alexander, 1950) that above pH 8·5 the chlorine is not effective in reducing shrinkage. Methods of rendering wool unshrinkable depend on disulphide bond breakdown in the surface layers of the fibres (Speakman, Nilssen & Elliott, 1938). Farnworth, Neish & Speakman (1949) have shown that fibres in which lanthionine had been formed could not be rendered unshrinkable. The sharp decrease in resistance to felting above pH 8·5 is in agreement with lanthionine formation. This is also supported by the high tensile strength of such fibres (see Table 3).

Oxidation in acid solution

The relevant observations on oxidation in acid are summarized on p. 134.

On oxidation the disulphide bonds in the wool are broken, yielding the theoretical amount of cysteic acid on acid hydrolysis without further oxidation, although (see p. 132) no free sulphonic acid groups are present in the wool before hydrolysis. In addition to the evidence from cationic exchange it was found that the oxidized wool has the same affinity for dyes as the untreated wool, whereas wool containing charged



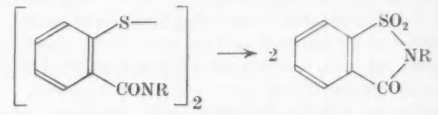
acidic groups has been found to repel dye anions (Lemin & Vickerstaff, 1946).

It was found that the soluble peptides obtained by ammonia extraction of wool oxidized with peracetic acid or chlorine were rich in cysteic acid, but the insoluble portion on hydrolysis contained little cysteic acid, no lanthionine and no sulphate. Total sulphur analyses showed, however, that no appreciable loss in sulphur had occurred during alkaline extraction (see Table 7). The elasticity measurements (Tables 3 and 6) on oxidized fibres support disulphide bond breakdown. There is a considerable reduction in work required for a given extension, but from the solubility in weak alkali (Fig. 1) it is seen that the oxidized residues are firmly held to the chains. It appears that in the keratin molecule, two adjacent cysteic acid groups cannot be formed on the disruption of the disulphide bond, probably owing to electrical repulsion. If the chains can move apart in the alkali solution after complete oxidation with peracetic acid, this restriction no longer exists. The soluble (90 %) portion was shown to contain 3·7 % cysteic acid sulphur after normal hydrolysis. Product II was not formed under these conditions. It is unlikely that intermediate oxidation products are formed to any extent (Harris & Smith, 1937), as these would not be quantitatively converted to cysteic acid on hydrolysis: e.g. the disulphone is hydrolysed to one mol. of sulphinic and one mol. of sulphonic acid (Lavine *et al.* 1934).

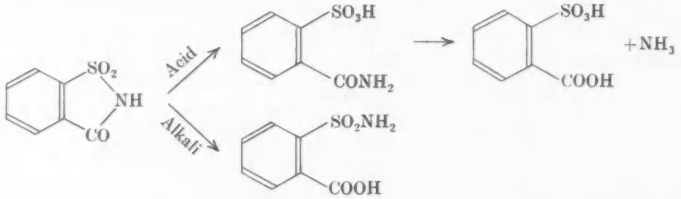
The most likely possibility when the stereochemical arrangement of the polypeptide chains is considered, appears to be intramolecular cyclization to give a compound which readily hydrolyses in acid to give cysteic acid. In alkaline solution, however, an alternative reaction occurs, leading to a very stable sulphur-containing compound. On this basis the set of reactions is suggested as probable (see p. 135).

The oxidation intermediate (I) is a mixed imide containing a sulphonic and a carboxylic acid residue with the N atom highly activated by the two negative groups $>CO$ and $>SO_2$ and is therefore so unstable that it cannot exist in solution. With acid it hydrolyses as a typical amide, the S—N bond breaking to give cysteic acid. In alkaline solution, however, hydroxyl ions attack the OC—N linkage giving a free carboxyl group (II). Hydrolysis of (II) with 15 % hydrochloric acid yields a *N*-β-sulphoalanyl amino-acid complex (III) which is partially hydrolysed under vigorous conditions to give cysteic acid and the amino-acid which was originally in the wool linked to the cystine residue through its carboxyl group.

This proposed set of reactions closely follows that of the formation and hydrolysis of saccharin and its *N*-substituted derivatives. Thus, oxidation of the *N*-substituted 2:2′-dithiobenzamide gives a substituted saccharin (McLelland, Warren & Jackson, 1929).



This behaves differently towards acid and alkaline hydrolysis, S—N bond fission occurring in the former case, C—N bond fission in the latter.



In the acid hydrolysis the amide formed is easily hydrolysed unlike the imide formed in wool.

The formation of the combined sulphonamide explains the observation that the tensile strength of the fibres remains the same after initial oxidation and subsequent mild alkali treatment. The sulphonamide forms without main chain breakdown although an extra group is incorporated. After treatment with ammonia, fibres oxidized with peracetic acid, but not untreated fibres, had an increased acid-combining capacity (see Table 8). Sulphonic acid groups cannot be responsible for this increase since they would not be titrated by 0·05 N-hydrochloric acid. Also, since the wool had been equilibrated with N-potassium chloride and the titration was carried out in the presence of potassium chloride, no acid could have combined by ion exchange. The increased titration of the wools can therefore be assumed to be due to the formation of free carboxyl groups. Thus, 60 % of the theoretical yield of carboxyl groups formed in the conversion of the combined sulphocarboxylic imide to the sulphonamide has been detected.

The conversion of the substituted sulphonamide (III) to cysteic acid by hydrolysis is only achieved

with difficulty. Sulphonyl derivatives of amino-acids have been used for end group determinations in proteins. Thus, Gurin & Clarke (1934) found that hydrolysis with 15% hydrochloric acid for 5 days at 90–100° resulted only in a 28% yield of the amino-acid from benzenesulphonylalanine. Helferich & Grünert (1940) found methanesulphonyl derivatives of amino-acids and peptides to be extremely resistant to both acid and alkaline hydrolysis. In the present investigation it was found that short hydrolysis (5 hr.) with concentrated hydrochloric acid or prolonged hydrolysis (approx. 4 days) with 25% hydrochloric acid resulted in a considerable increase in cysteic acid (see p. 133). More drastic conditions were shown to lead to the destruction of cysteic acid so that quantitative conversion of the sulphonamide to the cysteic acid is impossible.

An attempt to isolate the stable sulphur compounds from the hydrolysate of wool which had been oxidized and extracted with alkali was made using a weakly basic ion-exchange column, since the sulphonamides postulated would be expected to be acidic. In this way a solution was obtained which contained no free amino-acids other than aspartic and glutamic acids. When this solution was hydrolysed under fairly drastic conditions one-dimensional chromatograms showed the presence of all the amino-acids found in wool, including proline, together with cysteic acid. A noticeable feature was the high proportion of aspartic acid so obtained. That the amino-acids which appeared after strong hydrolysis were not derived from cysteic acid peptides stable to normal hydrolysis is shown by the fact that all the cysteic acid can be recovered by normal hydrolysis from wool which had been oxidized but not reacted with alkali. It is also unlikely that peptides of aspartic or glutamic acid would survive normal protein hydrolysis since Partridge & Davis (1950) and Bull (1949) have shown that these amino-acids are preferentially liberated during acid hydrolysis. These results agree with the general conclusions of Consden *et al.* (1949) and Consden & Gordon (1950), except that these workers failed to detect proline and aspartic acid combined with the cystine in wool.

Apart from the main reactions which have been fully discussed above, evidence for the limited formation of an intermediate cystine oxidation compound is obtained from cystine analyses of wool which has been treated with chlorine at pH 2 (see Table 9). There is also evidence that chlorination in acid may lead to cross linking. It is shown in Fig. 1 that wool, in which 90% of the disulphide bonds have been oxidized by peracetic acid, is almost completely soluble in ammonia. The small insoluble fraction which remains is the subcuticle membrane (Alexander & Earland, 1950*a*). The fibres, however, remain largely insoluble in ammonia until more than 80% of the disulphide bonds have been broken. However, wool oxidized to the same extent with chlorine is only soluble in ammonia to the extent of 70%. Moreover, while the wool oxidized with peracetic acid supercontracts, the chlorinated wool does not (Whewell & Woods, 1946). It is suggested that on chlorination in acid solution, new cross-linkages are formed which prevent supercontraction and increase the resistance to alkali.

SUMMARY

1. Oxidation of wool with solutions of potassium permanganate at pH 9·2 and sodium hypochlorite at pH 10 led to the formation of lanthionine in the cold from the reactive cystine fraction (approx. 30% of the total cystine).

2. Acidic solutions of potassium permanganate, which also react with only 30% of the total cystine, gave cysteic acid and sulphate.

3. On oxidation with acid solutions of chlorine and peracetic acid, which are capable of reacting with all the cystine, combined cysteic acid was not produced directly within the fibre although cysteic acid was produced almost quantitatively on acid hydrolysis. To explain this and other observations the formation of a combined heterocyclic mixed imide of a carboxylic and sulphonic acid respectively within the wool is postulated.

4. In order to interpret results obtained by treatment of wool oxidized with peracetic acid or chlorine at pH 2 with dilute alkali it is postulated that alkali hydrolyses this sulphocarboxylic imide across the CO—N bond to give a sulphonamide within the fibres. The formation of the sulphonamide explains the increase in acidity on alkali extraction.

5. Hydrolysis of the postulated sulphonamides by drastic treatment with hydrochloric acid led to the production of cysteic acid together with all the amino-acids of wool. This shows that cystine is combined with all the various amino-acids within the fibre.

6. Evidence for the limited formation of a partially oxidized product formed from cystine by a side reaction was obtained. As this reverts to cystine, at least partially, on treatment with alkali, it is suggested that this intermediate may be a sulphoxide.

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REFERENCES

Alexander, P. (1950). *J. Soc. Dy. Col., Bradford*, **66**, 349.
Alexander, P. & Earland, C. (1950*a*). *Text. Res. J.* **20**, 298.
Alexander, P. & Earland, C. (1950*b*). *Nature, Lond.*, **166**, 396.
Alexander, P. & Gough, D. (1951). *Biochem. J.* **48**, 504.
Alexander, P., Hudson, R. F. & Fox, M. (1950). *Biochem. J.* **46**, 27.
Bull, H. B. (1949). *Cold Spr. Harb. Sym. quant. Biol.* **14**, 2.
Consden, R. & Gordon, A. H. (1950). *Biochem. J.* **46**, 8.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1946). *Biochem. J.* **40**, 580.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1948). *Biochem. J.* **42**, 443.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1949). *Biochem. J.* **44**, 548.
Cuthbertson, W. R. & Phillips, H. (1945). *Biochem. J.* **39**, 7.
Dent, C. E. (1947). *Biochem. J.* **41**, 240.
Farnworth, A. J., Neish, W. J. P. & Speakman, J. B. (1949). *J. Soc. Dy. Col., Bradford*, **65**, 447.
Gurin, S. & Clarke, H. T. (1934). *J. biol. Chem.* **107**, 395.
Harris, M., Mizell, A. R. & Fourt, R. (1942). *Bur. Stand. J. Res., Wash.*, **29**, 73.
Harris, M. & Smith, A. L. (1937). *Bur. Stand. J. Res., Wash.*, **18**, 623.
Helferich, B. & Grünert, H. (1940). *Liebigs Ann.* **545**, 178.
Horn, M. J., Jones, D. B. & Ringel, S. J. (1941). *J. Biol. Chem.* **138**, 141.
Jones, J. H. (1943). *Association of Official Agricultural Chemists, Washington*, **26**, 182.
Kressman, T. R. E. & Kitchener, J. A. (1949). *J. chem. Soc.* p. 1190.
Lavine, T. F. (1936). *J. biol. Chem.* **113**, 583.
Lavine, T. F., Toennis, G. & Wagner, E. C. (1934). *J. Amer. chem. Soc.* **56**, 242.
Lemin, D. R. & Vickerstaff, T. (1946). *Symposium on Fibrous Proteins*, p. 129. Bradford: Society of Dyers and Colourists.
McLelland, E. W., Warren, L. A. & Jackson, J. H. (1929). *J. chem. Soc.* p. 1582.
Partridge, S. M. & Davis, H. F. (1950). *Nature, Lond.*, **165**, 62.
Polson, A., Mosley, V. M. & Wyckoff, R. W. C. (1947). *Science*, **105**, 603.
Shinohara, K. (1935). *J. biol. Chem.* **109**, 665.
Sookne, A. M. & Harris, M. (1937). *Bur. Stand. J. Res., Wash.*, **19**, 535.
Speakman, J. B. (1945). *J. R. Soc. Arts*, **93**, 603.
Speakman, J. B. (1947). *J. Text. Inst., Manchr*, **38**, T102.
Speakman, J. B. & Elliott, C. H. (1940). *J. chem. Soc.* p. 641.
Speakman, J. B., Nilssen, B. & Elliott, C. H. (1938). *Nature, Lond.*, **142**, 1035.
Stoves, J. L. (1946). *Symposium on Fibrous Proteins*, p. 58. Bradford: Society of Dyers and Colourists.
Whewell, C. S. & Woods, H. (1946). *Symposium on Fibrous Proteins*, p. 50. Bradford: Society of Dyers and Colourists.

The Metabolism and Glutamic Acid Content of Rat Brain in Relation to Thiopentone Anaesthesia

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It has recently been shown that the free glutamic acid content of the rat brain falls during insulin hypoglycaemia (Dawson, 1950). This observation suggests that in the physiological emergency produced by a shortage of glucose the brain oxidizes glutamic acid to gain high-energy phosphate bonds for metabolic activity. It is well known that during barbiturate anaesthesia the metabolic activity of the brain, as measured by its consumption of oxygen, slows down in parallel with the reduction of physiological activity (Etsten & Himwich, 1946; Himwich, Homburger, Maresca & Himwich, 1947). It has been suggested from the results of *in vitro* studies that this depressed metabolism is a consequence of inhibition of brain oxidations by the narcotic (Quastel & Wheatley, 1932). The present investigation was undertaken to determine whether the reduced metabolism of the brain during anaesthesia was associated with a change in the level of the free glutamic acid in the brain. It was found that in the rat during anaesthesia produced by sodium 5-ethyl-5-(1′-methylbutyl)-2-thiobarbiturate (thiopentone sodium) the free glutamic acid content of the brain was reduced by about 30%. Simultaneous observations also showed that thiopentone anaesthesia reduced the concentration of ammonia found in the brain and slightly increased the glutamine content. Observations were also made on the inhibition by thiopentone of the respiration of isolated rat-brain tissue, and the effect of the drug on some enzyme systems in brain tissue capable of producing free ammonia.

METHODS

Young male rats of the Wistar albino strain were used throughout. For the determination of free glutamic acid and glutamine in whole brain, rats weighing between 40 and 80 g. were killed by decapitation and their heads transferred to liquid oxygen within a few seconds. The subsequent procedure has been described previously (Dawson, 1950).

Glutamic acid and glutamine determinations. These substances were estimated by the microbiological method of Krebs (1948) with certain modifications (Dawson, 1950). With some cultures of *Clostridium welchii* S.R. 12 it was noticed that brain extracts gave a very slow evolution of CO_2 after the complete decarboxylation of glutamic acid and glutamine. This slow evolution of CO_2 occurred both aerobically and anaerobically. The substrate has not yet been identified, although it was not histidine. The results presented have, where necessary, been corrected for this factor.

Ammonia was estimated by the microdiffusion method of Conway (1947) with the modifications used by Richter & Dawson (1948).

Paper chromatography of the amino-acids in rat-brain extracts. One-dimensional filter-paper chromatograms of the free amino-acids in rat-brain extracts were prepared using phenol as the solvent. The rats were decapitated and the heads immediately immersed in liquid oxygen. The frozen whole brain was removed from the head, crushed, and the powder then shaken with 5 ml. of 12% (w/v) trichloroacetic acid solution contained in a weighed centrifuge tube. After reweighing, the suspension was centrifuged and 2 ml. of the supernatant was extracted twice with 5 ml. of ether. A sample of the ether-extracted solution was spotted on to a filter-paper sheet with an 'Agla' micrometer syringe. Brain extracts from anaesthetized and control animals were spotted in pairs and the volume of the sample (approx. 0·1 ml.) adjusted to correspond to a definite weight of brain. Glutamic acid standards were freshly prepared in 10% (w/v) trichloroacetic acid solution and extracted with ether in a similar way to the brain extracts. After chromatography overnight with redistilled phenol, the filter-paper sheets were dried in a current of air at room temperature to avoid loss of the amino-acids (Fowden & Penney, 1950). Thorough drying was found to be essential, as traces of phenol interfered with the subsequent development of the amino-acid spots with ninhydrin. The dried sheets were then sprayed with 0·1% ninhydrin dissolved in *n*-butanol. The resulting chromatogram of the amino-acids showed five distinct spots, the most prominent one containing the free glutamic acid. Although this main spot consisted largely of glutamic acid, an examination of two-dimensional chromatograms showed that it was composite and contained small quantities of other ninhydrin-reacting substances. In two pairs of rats amino-acid determinations on the 'glutamic acid' spots were performed by Mr G. Ansell. The chromatograms were sprayed with 0·1% NaOH to eliminate NH_3, and the amino-acid N estimated by the colorimetric method of Moore & Stein (1948).

Respiration of minced brain tissue. The direct method of Warburg was used to measure the O_2 uptake of minced brain tissue. Young rats (90–110 g.) were killed and the whole brain rapidly removed, chilled and finely minced with a razor. The minced brain was weighed and suspended in Krebs phosphate-Ringer (200 mg. brain tissue/ml.). Samples of the suspension (1 ml.) were pipetted into Warburg vessels already containing additional Ringer solution and glucose. Air was used in the gas space to avoid O_2 poisoning (Elliott & Libet, 1942; Mann & Quastel, 1946; Dickens, 1946). Respiration measured over the first hour amounted to approx. 1250 μl./g. wet wt./hr. In some experiments the O_2 consumption was measured in the presence of a substrate after a previous 3 hr. period of autoxidation (Quastel & Wheatley, 1932). In these measurements use was made of a modified Krebs phosphate-Ringer solution containing a high concentration of K; part of the NaCl being replaced by KCl. This high concentration of K tends to stabilize the inhibition of oxidations produced by barbiturates (Jowett & Quastel, 1937).

Thiopentone anaesthesia. A solution containing 10 mg./ml. of sodium 5-ethyl-5-(1′-methylbutyl)-2-thiobarbiturate (Pentothal, Abbott) was injected intraperitoneally. For *in vitro* experiments, freshly prepared sodium thiopentone solution was adjusted to the required pH with HCl and added to the Warburg vessel as a suspension of the free thiobarbituric acid. Purification of the free acid by a one-stage crystallization from ethanol-water had no effect on the melting point (156°) nor any apparent effect on the inhibition of brain respiration.

Glutaminase. The method of Krebs (1935) was used to determine glutaminase activity in a simple extract of whole rat brain. The NH_3 produced by incubation of the extract at 37° with glutamine (British Chemicals and Biologicals Ltd.) at pH 8·5 was measured by the Conway microdiffusion procedure.

Adenylic deaminase. Activity of this enzyme in extracts of rat brain was determined by a modification of the method of Conway & Cooke (1939). Incubation times of less than 1 hr. and a higher temperature (37°) were used. Adenylic acid (Roche) was added to give a final concentration of 0·6%.

RESULTS

The results of microbiological determinations of the ammonia, glutamine and free glutamic acid content of rat brain are presented in Table 1. The free glutamic acid present in a series of brains taken from rats anaesthetized with thiopentone was significantly less than that of a control series (by Student's t test, $P < 0.001$). The mean glutamine concentration in the brains of the anaesthetized rats was slightly raised, the increase being on the borderline of statistical significance (by Student's t test, $P = 0.05$). The sum of the glutamic acid present as the free acid and that as the amide was, however, significantly less in the anaesthetized rats ($P < 0.001$). This showed that the reduction in the free glutamic acid level could not be solely accounted for by the increased glutamine concentration. The ammonia content of the brain was lower in the rats killed during anaesthesia. It is known that part of this ammonia would be formed after death, since the ammonia content of rat brain increases very rapidly after decapitation (Richter & Dawson, 1948).

When rats are anaesthetized with barbiturates the body temperature gradually falls, unless heat is supplied to the animal. In the anaesthetized rats the rectal temperature fell well below the normal value, and more anaesthetized rats were therefore

Table 1. *Ammonia, glutamine and free glutamic acid content of the brain in normal and anaesthetized rats*

	Dose of thiopentone (mg./kg.)	Duration of anaesthesia (min.)	NH_3 (mg./100 g. tissue)	Glutamine (mg. glutamic acid/100 g. tissue)	Free glutamic acid (mg./100 g. tissue)	Free glutamic acid + glutamine (mg. glutamic acid/100 g. tissue)
Controls	—	—	0·8	84	154	238
	—	—	1·1	84	178	262
	—	—	0·7	58	187	245
	—	—	0·6	85	184	269
	—	—	0·6	83	171	254
	—	—	0·5	77	157	234
		Means	**0·7**	**78**	**172**	**250**
Anaesthetized	60	65	0·4	84	108	192
	60	15	0·4	81	134	215
	50	70	0·3	89	123	212
	50	45	0·3	96	121	217
	60	55	—	81	115	196
	70	55	—	93	93	186
	65	65	0·2	101	112	213
		Means	**0·3**	**89**	**115**	**204**
Anaesthetized and body temperature maintained	65	55	—	67	173	240
	75	55	—	78	146	224
	65	> 30	0·5	71	148	219
	65	> 30	0·4	83	143	226
	65	75	0·5	87	151	238
		Means	**0·5**	**77**	**152**	**229**

Table 2. *Ammonia, glutamine and free glutamic acid content of the rat brain after electrical stimulation and variation of the environmental temperature*

	No. of rats	NH_3 (mg./100 g. tissue)	Glutamine (mg. glutamic acid/100 g. tissue)	Free glutamic acid (mg./100 g. tissue)	Free glutamic acid + glutamine (mg. glutamic acid/100 g. tissue)	Remarks
Controls (mean of 6 rats)	6	0·7 (0·5–1·1)	78 (58–85)	172 (154–187)	250 (234–269)	—
Electrical stimulation (40 V., 1–2 sec., 50 cyc./sec. a.c.)	3	0·8	81	165	246	Killed in convulsions
		1·3	83	164	247	
		0·7	64	183	247	
High enviromental temp. (37°; 1·5 hr.)	2	1·0	88	179	267	Rectal temp. 39°
		0·8	73	177	250	
Low environmental temp. (−5°; 1·5 hr.)	2	1·3	83	185	268	Rectal temp. below 34°
		0·6	68	165	233	

examined, in which the body temperature had been maintained. This was conveniently done by keeping them in a biological incubator at 37° during the narcosis. Estimations of the free glutamic acid, glutamine and ammonia present in the brain showed that maintenance of the body temperature during anaesthesia reduced the amount by which the glutamic acid level in the brain fell (Table 1). However, the levels of the free glutamic acid and of glutamic acid + glutamine were still both significantly reduced below the control values. The mean level of glutamine in these rats was normal. It is probable that maintenance of the body temperature during thiopentone anaesthesia reduces the severity of the anaesthesia by aiding detoxication of the drug. A similar result was noted during an investigation of the effect of pentobarbitone anaesthesia on the phosphorus metabolism of the brain (Dawson & Richter, 1950*a*). Further experiments were made in which young rats were subjected to high (37°) and low (−5°) environmental temperatures for 1·5 hr. before killing. Estimations of the glutamine and free glutamic acid content of the brain showed that these were within the normal range (Table 2).

In three experiments the concentrations of glutamine and free glutamic acid in the brain were measured in rats which had been killed during convulsions induced by electrical stimulation of the brain (Richter & Dawson, 1948). This increased activity of the brain over a short period of time had no effect on the level of glutamine or free glutamic acid in the organ. Haber & Saidel (1948) have reported

a lowering of the free glutamic acid content of the brain in rats killed during strychnine convulsions.

Confirmation of the reduced level of free glutamic acid in the rat brain during thiopentone anaesthesia was obtained by observation of amino-acid chromatograms of brain extracts. Samples of extracts prepared from normal and anaesthetized litter mates were spotted as pairs on filter-paper sheets and subjected to chromatography with phenol. After development of the chromatogram with ninhydrin, eight observers were asked to choose, from the intensity and area of the colour, which 'glutamic acid' spot of a given experimental pair contained the more amino-acid. The identity of the spots was unknown to the observers and if no difference could be detected between the spots the result was not recorded. Table 3 records the results obtained by the visual observation of seven pairs. In two pairs (nos. 4 and 5, Table 3) estimations of the amino-acid nitrogen of these spots were made; the values, in mg. N/100 g. brain, were 19·8 and 20·2 for the control animals and 12·4 and 11·0 for the anaesthetized animals. These results support the conclusions reached from the microbiological estimations.

Table 3. *Examination of the 'glutamic acid' spot on one-dimensional chromatograms of brain extracts from control and anaesthetized rats*

(Rats injected intraperitoneally with thiopentone sodium (60 mg./kg.) and killed after 1·2–1·7 hr. anaesthesia.)

Experimental pair no.	Visual observation of 'glutamic acid' spot* Control	Anaesthetized
1	8	0
2	5	1
3	7	0
4	2	1
5	8	0
6†	7	0
7	8	0

* Each experimental pair of spots was judged for greater amino-acid content by eight observers. Columns record the number of these observers who considered that one spot of an experimental pair contained more amino-acid than the other.

† 7 observers.

Thiopentone inhibition of brain respiration. As the possibility exists that the reduced glutamic acid content of the rat brain during thiopentone anaesthesia is connected with the lowered glucose utilization of the brain, it was decided to investigate the effect of the drug on the respiration of isolated brain tissue with various substrates. It was found that the oxygen consumption of whole rat brain which had been minced and suspended in a glucose-Ringer solution was reduced by low concentrations of thiopentone (Table 4). Thiopentone as the free thiobarbituric acid is only slightly soluble in water, and measurements showed that the maximum solubility in a phosphate buffer of pH 7·4 at 37° was not greater than 0·015 %. It was found that on the addition of thiopentone suspensions to produce a concentration higher than 0·015 % the respiration of minced brain tissue was still further reduced. A possible explanation of this result is that there is an absorption of thiopentone into the lipids of brain tissue. The inhibition by thiopentone of the respiration of isolated rat-brain tissue has also been determined by Fuhrman & Field (1943). These workers investigated the inhibition over a somewhat higher range of drug concentrations, the narcotic being added as the alkaline solution of the sodium salt. They found that at high concentrations (0·15 %) of thiopentone the respiration of rat-brain slices was inhibited by about 80 %.

Table 4. *Inhibition of the respiration of minced rat-brain tissue by thiopentone*

(Results are the mean of two determinations. Each Warburg vessel contained 200 mg. minced brain in 1·8 ml. Krebs phosphate-Ringer solution. Glucose concentration 0·015 M. Temp. 37°.)

Thiopentone added (%)	Inhibition of respiration during 1st hr. (%)
0·08	21
0·04	19
0·02	15
0·01	10
0·005	8
0·001	0

The oxidation of glutamic acid by brain tissue is known to be inhibited by low concentrations of barbiturates (Quastel & Wheatley, 1932). Table 5 records the effect of 0·04 % thiopentone on the stimulation of respiration produced by adding glutamate to minced brain tissue, after 3 hr. of previous autoxidation. In the initial stages of this autoxidation without any added substrate, the oxygen uptake of minced brain tissue was reduced by thiopentone. However, after 2 or 3 hr. the respiration was not inhibited by the drug and was sometimes even slightly higher than that of the control.

The oxidation of α-ketoglutaric acid by minced rat-brain tissue was also found to be inhibited by thiopentone (Table 5). When, however, glucose was added to minced brain tissue which had been allowed to autoxidize for 3 hr. the resulting stimulation of respiration was almost unaffected by the presence of thiopentone (Table 5). In experiments in which glucose was present from the beginning the respiration was still inhibited by thiopentone during the fourth hour (Table 6), the magnitude of the inhibition being usually increased, as is common with other barbiturates (Jowett & Quastel, 1937). It would appear, therefore, that the preliminary autoxidation of the brain tissue affects the oxidative

Table 5. *Inhibition by thiopentone of oxidations performed by minced rat-brain tissue after autoxidation for 3 hr.*

(All results are the mean of two determinations performed on different rat brains. Each Warburg vessel contained: 1 ml. phosphate buffer pH 7·4; 0·8 ml. Krebs phosphate-Ringer solution with high K content (final concentration 0·015 M); 200 mg. minced brain. Substrate 0·3 ml. in side arm, previously brought to pH 7·4 with NaOH. Centre cup: 0·2 ml. 5% KOH. Temp. 37°.)

Substrate	Thiopentone added (%)	Inhibition in 1st hr. in absence of added substrate (%)	Inhibition in 1st hr. in presence of added substrate (%)	Inhibition in 2nd hr. in presence of added substrate (%)
Glutamate (0·03 M)	0·04	22	42	41
α-Ketoglutarate (0·03 M)	0·04	23	34	32
Glucose (0·03 M)	0·04	21	3	−5

Table 6. *Typical experiment showing the effect of thiopentone and glutamate on the respiration of minced rat-brain tissue*

(200 mg. minced rat brain in 1·8 ml. of Krebs phosphate-Ringer solution. Temp. 37°.)

Substrate	Oxygen consumed (μl.) 1st hr.	2nd hr.	3rd hr.	4th hr.
None	227	152	92	54
Glucose (0·015 M)	260	234	197	155
Glucose (0·015 M) + sodium glutamate (0·005 M)	275	234	176	116
None + 0·04% thiopentone	162	132	88	53
Glucose (0·015 M) + 0·04% thiopentone	205	189	148	105
Glucose (0·015 M) + sodium glutamate (0·005 M) + 0·04% thiopentone	205	169	132	86

systems so that the thiopentone-inhibited step is no longer a limiting factor of glucose oxidation.

In some further experiments the respiration of minced rat brain tissue was measured with a substrate of glucose (0·015 M) plus glutamate (0·005 M). It was found that the addition of thiopentone caused a degree of inhibition of the oxygen uptake which was approximately the same as when glucose was the only substrate. The presence of the glutamic acid slightly accelerated the oxygen uptake during the first hour, but the rate of fall of the respiration was then faster in the presence of glutamic acid and glucose than with glucose alone (Table 6). Dickens (1946) has observed that the presence of glutamic acid accelerates the poisoning of brain slices by high oxygen pressures. It is therefore possible that glutamic acid can also accelerate the oxygen poisoning of minced brain tissue by air.

The effect of thiopentone on the glutaminase and adenylic deaminase of rat brain. The reduced ammonia content found in the brains of anaesthetized rats suggested the possibility of an interference with enzyme systems which can produce ammonia in brain tissue. It was found that the spontaneous liberation of ammonia in buffered extracts of whole rat brain was unaffected by thiopentone (Table 7). The hydrolysis of glutamine by the glutaminase present in rat-brain extracts was also not inhibited by thiopentone (Table 7). The deamination of adenylic acid by brain extracts was in all experiments slightly reduced on the addition of 0·04% thiopentone (Table 7). The inhibition appeared to increase with time and was also shown when the incubation temperature was 17 or 30°.

Table 7. *Effect of thiopentone on some enzyme systems in brain capable of producing free ammonia*

Enzyme system	Thiopentone added (%)	Inhibition during 1st 30 min. (%)
Brain extract: spontaneous NH_3 formation	0·04	0
Glutaminase of brain extract	0·04	0
Adenylic deaminase of brain extract	0·04	14*

* Mean of three determinations on different rat brains.

Initial results show that adenylic deaminase of brain is also poisoned by barbitone, although urethane has no action on the enzyme.

DISCUSSION

The present results clearly indicate that the free glutamic acid content of the brain is reduced in rats anaesthetized with thiopentone. The estimations of glutamic acid were made after decapitation of the animals and the rapid transference of the head to liquid oxygen to stop the post-mortem biochemical activity. Unless it is believed that the difference in the glutamic acid level became apparent in the brief interval between death and the termination of enzymic activity the change described must be regarded as having occurred *in vivo*.

During anaesthesia it has been shown that the consumption of oxygen by the brain *in vivo* is lowered (Himwich *et al.* 1947), and it seems possible that the reduction of the glutamic acid content is associated with this depressed metabolism. Thus, glutamic acid is closely connected with the oxidative degradation of glucose through the glutamic dehydrogenase system. It has been suggested that one of the physiological functions of glutamic acid in brain is to act as a metabolic buffer of the tricarboxylic acid cycle, assuring an optimal functional rate of the cycle at the α-ketoglutaric acid step (Waelsch, 1949).

It is also possible that a depression of the respiration would allow an actual loss of glutamic acid from the brain to the blood stream. In this connexion it has been shown by Stern, Eggleston, Hems & Krebs (1949) that the maintenance of a high glutamic acid content in isolated slices of brain is dependent upon respiration and energy-giving reactions.

In considering these possible explanations of the reduced glutamic acid level in the brain during thiopentone anaesthesia it becomes desirable to know more about the mechanism by which the barbiturates produce their narcotic activity. It has been suggested by Quastel & Wheatley (1932) that barbiturates cause anaesthesia by an actual inhibition of the oxidations occurring in nervous tissue. This theory, and the alternative viewpoint that the reduced metabolism of the nervous system is a secondary result of the depression of nervous activity, have recently received considerable attention (Gerard, 1947; Butler, 1950).

From the present measurements it can be concluded that the respiration *in vitro* of minced rat brain tissue can be inhibited by low concentrations of thiopentone. In some further experiments it was found that an injection of 30 mg. sodium thiopentone/kg. would just lightly anaesthetize young rats. Assuming the drug was equally distributed through the soft tissues of the body (Butler, 1950) the concentration of thiobarbiturate in the brain *in vivo* would only just inhibit the respiration of minced brain tissue *in vitro*. However, the drawing of definite conclusions from these results is prevented by a consideration of the different conditions under which the brain respires *in vitro* and *in vivo*. Moreover, the possibility of localized regions of barbiturate concentration and inhibition in the brain *in vivo* cannot be excluded. Additional evidence which bears on this problem is provided by the observation that the level of high-energy phosphoric esters in the brain actually increases during barbiturate anaesthesia (Stone, 1940; Dawson & Richter, 1950*b*). This observation is not consistent with the theory that the actual cause of anaesthesia is a primary inhibition of brain oxidations, if it is assumed that carbohydrate metabolism is related to nerve function through the energy-rich phosphoric esters (Dawson & Richter, 1950*b*; Buchel & McIlwain, 1950).

It seems unlikely, therefore, that the fall of the glutamic acid level in the brain occurring during thiopentone anaesthesia is connected with a physiological necessity for the production of energy-rich phosphate bonds. If the glutamic acid lost is metabolized by oxidation it must be considered as a response of the reversible glutamic dehydrogenase system to the reduced production of α-ketoglutaric acid by glucose degradation.

The reduced ammonia content of the anaesthetized brain found in the present investigation and in previous work (Richter & Dawson, 1948) may be associated with the slightly raised glutamine concentration. The synthesis of glutamine is known to require high-energy phosphate bonds (Elliott, 1948), which are present in the brain during anaesthesia at an even higher concentration than normal. Glutamine hydrolysis is not likely to occur in response to the reduced glutamic acid level, as brain glutaminase shows little activity at a physiological pH (Krebs, 1935). The present observations also indicate that the enzyme in the brain deaminating adenylic acid can be poisoned *in vitro* at a low concentration of thiopentone. This result is, however, of doubtful physiological significance as it was shown by Kerr (1942) that measurable free adenylic acid was present in the brain only after some minutes of post-mortem enzymic activity.

SUMMARY

1. The free glutamic acid content of rat brain estimated after decapitation is reduced in animals anaesthetized with thiopentone.

2. The glutamine content of the brain is slightly increased in anaesthetized rats, but this accounts for only part of the fall in the glutamic acid level.

3. Maintenance of the body temperature of the rats during narcosis decreases the amount by which the glutamic acid level in the brain is reduced.

4. The glutamine and glutamic acid levels in the rat brain are not significantly changed from the normal values by alteration of the environmental temperature or by electrical stimulation of the brain.

5. The inhibition of the respiration of minced rat brain tissue by low concentrations of thiopentone has been measured.

6. Thiopentone anaesthesia reduces the level of free ammonia found in the rat brain.

7. Adenylic deaminase present in brain extracts is slightly poisoned by 0·04% thiopentone, while glutaminase is unaffected.

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REFERENCES

Buchel, L. & McIlwain, H. (1950). *Nature, Lond.*, **166**, 269.
Butler, T. C. (1950). *Pharm. Rev.* **2**, 121.
Conway, E. J. (1947). *Micro-diffusion Analysis and Volumetric Error*. London: Crosby Lockwood.
Conway, E. J. & Cooke, R. (1939). *Biochem. J.* **33**, 479.
Dawson, R. M. C. (1950). *Biochem. J.* **47**, 386.
Dawson, R. M. C. & Richter, D. (1950*a*). *Proc. roy. Soc.* B, **137**, 252.
Dawson, R. M. C. & Richter, D. (1950*b*). *Amer. J. Physiol.* **160**, 203.
Dickens, F. (1946). *Biochem. J.* **40**, 145.
Elliott, K. A. C. & Libet, B. (1942). *J. biol. Chem.* **143**, 227.
Elliott, W. H. (1948). *Nature, Lond.*, **161**, 128.
Etsten, B. & Himwich, H. E. (1946). *Anesthesiology*, **7**, 536.
Fowden, L. & Penney, J. R. (1950). *Nature, Lond.*, **165**, 846.
Fuhrman, F. A. & Field, J. (1943). *J. Pharmacol.* **77**, 392.
Gerard, R. W. (1947). *Anesthesiology*, **8**, 453.
Haber, C. & Saidel, L. (1948). *Fed. Proc.* **7**, 47.
Himwich, W. A., Homburger, E., Maresca, R. & Himwich, H. E. (1947). *Amer. J. Psychiat.* **103**, 689.
Jowett, M. & Quastel, J. H. (1937). *Biochem. J.* **31**, 565.
Kerr, S. E. (1942). *J. biol. Chem.* **145**, 647.
Krebs, H. A. (1935). *Biochem. J.* **29**, 1951.
Krebs, H. A. (1948). *Biochem. J.* **43**, 51.
Mann, P. J. G. & Quastel, J. H. (1946). *Biochem. J.* **40**, 139.
Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
Quastel, J. H. & Wheatley, A. H. M. (1932). *Proc. roy. Soc.* B, **112**, 60.
Richter, D. & Dawson, R. M. C. (1948). *J. biol. Chem.* **176**, 1199.
Stone, W. E. (1940). *J. biol. Chem.* **135**, 43.
Stern, J. R., Eggleston, L. V., Hems, R. & Krebs, H. A. (1949). *Biochem. J.* **44**, 410.
Waelsch, H. (1949). *Lancet*, **2**, 1.

The Composition of some Microbial Deoxypentose Nucleic Acids

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Recent advances in nucleic acid chemistry, principally through the application of chromatographic techniques, have led to the abandonment of the tetranucleotide hypothesis and the discovery that both deoxypentose nucleic acids (DNA) and ribonucleic acids (RNA) show a range of compositions characteristic of their source. Because of the close association of DNA with the chromatin material of the nucleus, comparative studies on the structure of DNA from different organisms have a special biological significance. Analyses by Chargaff and co-workers have led to the conclusion that, among animals, the composition of DNA (i.e. the molar proportions of the purine and pyrimidine bases) is characteristic of the species source and constant for different tissues of one species, and that DNA from bacteria also shows characteristic differences in composition (Chargaff, 1950; Chargaff, Zamenhof, Brawerman & Kerin, 1950). Results obtained in this laboratory with improved chromatographic techniques and methods of hydrolysis, while in general agreement with these conclusions, have also led to the recognition of the pyrimidine, 5-methylcytosine, as a constituent of DNA from animals and higher plants; this is apparently lacking from nucleic acids of microbial origin (Wyatt, 1950, 1951*a*, *b*). It is apparent that in molecules so large as those of the nucleic acids many possibilities exist for structural differences which do not involve differences in nucleotide composition; nevertheless it is only through such quantitative analysis that an approach may at present be made to the comparison of different nucleic acids. We have therefore extended these analyses to include some bacterial and virus deoxypentose nucleic acids, and shall now describe the determination of the purine and pyrimidine composition of DNA from *Mycobacterium tuberculosis*, *Escherichia coli*, the two *E. coli* bacteriophages T_2 and T_5, and the virus causing polyhedral disease in gipsy-moth (*Lymantria dispar* L.) caterpillars.

It is now recognized that all bacteria which have been examined contain both RNA and DNA, and there is evidence that the DNA is confined to the bacterial nucleus (Robinow, 1945). *E. coli*, which contains about 4% (dry wt.) of DNA, was chosen in order to permit comparison of the nucleic acids in a virus-host system. The tubercle bacillus was of particular interest because of the reported presence of 5-methylcytosine in its nucleic acid (Johnson & Coghill, 1925), although Vischer, Zamenhof & Chargaff (1949) had been unable to confirm this claim.

The bacteriophages and the polyhedral viruses of insects are convenient for nucleic acid analysis

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because of their relatively high content of DNA, and are, incidentally, the only viruses which have been shown conclusively to contain only nucleic acid of the deoxypentose type. The corresponding claim for certain animal viruses (e.g. vaccinia; Hoagland, Lavin, Smadel & Rivers, 1940) has been based on the properties of nucleic acid isolated with the use of hot 5% (w/v) aqueous sodium hydroxide, which would eliminate by hydrolysis any RNA that had been present. There has been controversy over the possible presence of RNA in the bacteriophages T_2 and T_6; recently, however, Cohen & Arbogast (1950) have shown by serological methods that RNA in T_2, T_4 and T_6 preparations is associated with material antigenically related to the host and that RNA is almost certainly not a constituent part of these viruses. The virus which may be isolated from gipsy-moth polyhedral bodies has been reported to contain about 14% of DNA, sufficient to account for its total phosphorus content (Bergold, 1947, 1950; Bergold & Pister, 1948).

MATERIALS

Mycobacterium tuberculosis

Samples of dried bacterial cells of human and bovine strains were obtained through the courtesy of the Wellcome Research Laboratories.

Escherichia coli

The mutant of the strain sensitive to the T series of bacteriophages designated as B/r (Witkin, 1946) was grown on nutrient broth (Lemco, 3 g.; peptone, 10 g.; NaCl, 5 g.; distilled water, 1 l.; pH 7·5) and on a synthetic medium of the following composition: sodium lactate, 10 g.; NH_4Cl, 1 g.; K_2HPO_4, 0·7 g.; KH_2PO_4, 0·3 g.; Na_2SO_4, 0·1 g.; $MgSO_4.7H_2O$, 0·01 g.; distilled water, 1 l. Stock cultures were subcultured daily on each medium. For the nucleic acid preparations 36 or 48 hr. cultures, grown on agar slopes in Roux flasks at 37° and in the stationary phase of growth, were rapidly washed three times in 0·9% (w/v) aqueous NaCl, twice in 90% (v/v) aqueous ethanol and in ethanol, suspended in ether for 30 min., centrifuged, and dried at 105° for 6 hr.

Bacteriophages T_2 *and* T_5

The bacteriophages T_2 and T_5 (Demerec & Fano, 1945) were grown on the B/r strain of *E. coli*. Stock suspensions were titrated and kept at 4°. The usual plaque-counting method of assay was used throughout the experiments. The procedure for growth and purification of the bacteriophages followed closely that used by Putnam, Kozloff & Neil (1949) for the isolation of the bacteriophage T_6. Broth lysates were prepared by inoculating a young aerated culture at 37° with a small number of bacteriophage particles and incubating for 8–16 hr. Here several cycles of adsorption and lysis were involved. Synthetic-medium lysates were prepared by 'single-stage growth'. To an aerated culture, containing about $1–3 \times 10^8$ bacteria/ml., bacteriophage was added to give 1–5 particles/bacterium and incubation continued for about 12 hr. The bacteriophage was isolated by differential centrifugation. The lysate was centrifuged at 3000 rev./min. to remove bacterial debris, and the supernatant passed through the Sharples supercentrifuge at 24,000 rev./min. sufficiently slowly to sediment over 95% of the virus. The pellet was suspended in a small volume of 0·85% (w/v) aqueous NaCl, centrifuged at low speed (15 min. at 3000 rev./min.) to remove insoluble material, and the virus again sedimented on the Sorvall SS1 angle centrifuge at 12,000 rev./min. for 1 hr. After resuspension in saline, more debris and aggregated material were removed by low-speed centrifuging.

As the purification of bacteriophage T_5 has not been described in the literature we record here some of the characteristics of our preparations. The ultraviolet absorption spectrum in neutral solution shows a peak at 260 mμ. and trough at 240 mμ., and is very similar to the absorption curves of T_2 and T_6 bacteriophages. The preparations, both from broth and the synthetic medium, were analysed for total N and P and assayed for infective particles. These results are shown in Table 1. The quantity of N/infective particle is higher than that reported for T_2 and T_6 bacteriophages ($1·3 \times 10^{-16}$ g. N/infective particle) and corresponds with the greater diameter of T_5 particles as shown by measurements on electron micrographs (Putnam, 1950). The mean diameter of T_5 given by Putnam is 90 mμ., while that for a dry particle calculated from the ratio N/infective particle, assuming an N content of 16% and a specific volume identical with that of T_2 and T_6 (0·66), is 80–85 mμ. Considering the errors involved in the various measurements the agreement is reasonably good.

Gipsy-moth polyhedral virus

Viruses causing the polyhedral diseases of insects were first shown by Bergold (1947) to have the form of rod-shaped particles which can be liberated from the polyhedra by dissolving the surrounding 'polyhedral protein' in dilute alkali. These polyhedra are obtained from infected caterpillars, in which they are freed in great abundance on lysis of the tissues at death, and can be purified and washed by centrifugation. The diseased gipsy-moth caterpillars (*Lymantria dispar* L.) which provided polyhedra for the present work were collected from an infestation in Slovakia in June, 1949. The polyhedra were purified centrifugally, and repeatedly washed in 0·9% (w/v) aqueous NaCl until a white suspension was obtained which showed very few bodies other than polyhedra on examination with the dark-field microscope. After the final sedimentation from saline, the polyhedra were suspended in distilled water and stored at −10° until needed.

Table 1. *Characteristics of the* T_5 *bacteriophage preparations*

	g. N/infective particle ($\times 10^{-16}$)	N/P ratio (by wt.)	Extinction coefficient at 260 mμ. of a solution containing 1 g. atom N/l.
Broth preparations (mean of two preparations)	3·3	3·55	1280
Synthetic-medium preparation	3·9	3·35	1180

Virus was prepared from the purified polyhedra by the following procedure, which is essentially that of Bergold (1947). Polyhedra were suspended at a concentration of about 5 mg./ml. in a solution containing Na_2CO_3, 0·008 M, and NaCl, 0·05 M, and the mixture was allowed to stand at room temperature for about 3 hr. Examination with the dark-field microscope then showed that almost all of the polyhedra had dissolved, leaving free virus particles and empty skins or ghosts which represent the insoluble surface layer of the polyhedral bodies. The suspension was centrifuged at 6000 rev./min. for 6 min. on the Sorvall angle centrifuge to remove undissolved polyhedra, most of the skins, and any bacteria or other gross impurities. The yield of virus could be slightly increased by suspending and resedimenting this material. The supernatant liquid was then centrifuged at 9600 rev./min. for 60 min., and the virus sedimented as a translucent, bluish-white, birefringent pellet, on top of which was a less compact brownish layer consisting mainly of polyhedral skins. This impurity could be washed off, with the loss of some virus, by a carefully directed stream of water from a pipette. The virus was suspended in distilled water (about one-eighth of the volume of the original solution) and again sedimented for 60 min. at 9600 rev./min. It was then suspended in a small volume of water, and centrifuged briefly (about 2 min. at 5000 rev./min.) to remove larger particles and aggregated material.

It has not been possible to test the infectivity of such preparations in this laboratory. Examination of several of them with the electron microscope (by Dr V. E. Cosslett in the Cavendish Laboratory, Cambridge) showed that they consisted mainly of rod-shaped particles about 30 × 300 mμ. in size, occurring both singly and in bundles, and similar to those which Bergold has identified as the virus. Analysis of several preparations gave mean values N, 15·1 % and P, 1·77 % of the dry wt. (drying at 110°). The P content is rather higher than that reported by Bergold (1·33 %) and appears to reflect differences in the DNA content of his preparations and ours.

METHODS

The isolation of the nucleic acids

The procedures described by Mirsky & Pollister (1946) for the isolation of deoxyribonucleoproteins from animal and plant tissues are often ineffectual with bacteria, possibly because of the impermeability of the cell wall. It has been shown in this laboratory (Wyatt, 1951*b*) that treatment with N-NaOH at 37° does not change the purine and pyrimidine composition of DNA, although RNA is completely hydrolysed to nucleotides (Schmidt & Thannhauser, 1945). This method was therefore used to prepare the nucleic acids from both bacteria and viruses. The material was incubated for 16–20 hr. at 37° in a small volume of N-NaOH. Insoluble material was removed by centrifuging, the supernatant liquid brought to pH 4 with acetic acid, and one volume of ethanol added. The precipitate, containing DNA, was collected by centrifugation, dissolved in dilute NaOH or alkaline M-NaCl, and protein removed by gel formation on shaking with a mixture of 8 parts of $CHCl_3$ and 1 part of commercial octanol (v/v) (Sevag, Lackman & Smolens, 1938). A further precipitation with acetic acid and ethanol completed the purification. Nucleic acid thus prepared was free of RNA and contained very little protein, but when derived from bacteria usually contained considerable extraneous material apparently polysaccharide in nature.

Analysis of the nucleic acids

The purine and pyrimidine composition of the nucleic acids was determined by techniques which have already been fully described (Markham & Smith, 1949; Wyatt, 1951*b*). Following hydrolysis either with pure formic acid (175° for 30 min.) or with 72 % (w/w) aqueous $HClO_4$ (100° for 60 min.), the bases were separated on paper chromatograms using as the solvent 65 % (v/v) aqueous *iso*propanol 2·0 N with respect to HCl. They were then eluted and estimated spectrophotometrically.

For the bacterial nucleic acids, which were contaminated with carbohydrate, hydrolysis with $HClO_4$ was found advantageous, since the use of formic acid leaves extraneous absorbing materials which interfere with chromatography. These are largely destroyed by $HClO_4$, and a cleaner chromatogram results.

With viruses such as bacteriophages and insect viruses (which contain a high proportion of nucleic acid all of one type, and little or no carbohydrate other than that of the nucleic acid) it was found that good purine-pyrimidine chromatograms can be obtained without isolation of the nucleic acid, by hydrolysis of the whole virus. Although such chromatograms are heavily loaded with amino-acids, these do not interfere appreciably with estimation of the nucleic acid bases. Tryptophan is destroyed by acid hydrolysis and thus only tyrosine has sufficiently intense ultraviolet absorption to show on the prints of the chromatograms; with the solvent used it moves in a position between cytosine and uracil, where it does not interfere. This method was used in some of the analyses of the polyhedral virus, and gave proportions of the bases similar to those found from purified virus nucleic acid.

In these analyses the information considered of interest was the relative proportions of the purine and pyrimidine components of the nucleic acids. As the reliability of the analytical procedures had already been established, N and P analyses were made only on those preparations of which sufficient was available.

Elementary analyses

N was estimated by a micro-Kjeldahl technique (Markham, 1942), and P by the colorimetric method of Allen (1940).

RESULTS

All the bacterial and virus DNA's analysed contained the purines adenine and guanine and the pyrimidines cytosine and thymine. 5-Methylcytosine could not be detected in any of these nucleic acids, even when the appropriate bands from large chromatograms were eluted, concentrated and run in a different solvent, a method which is capable of detecting a pyrimidine occurring in amounts as small as 0·05 % of a nucleic acid (Wyatt, 1951*a*).

The molar proportions of the four bases in each nucleic acid are presented in Table 2. Each analysis is based on three or more replicate sets of spots on the chromatogram. The number of preparations of each nucleic acid analysed and the method of hydrolysis used are stated and the mean results given.

Table 2. *Proportions of purines and pyrimidines in deoxypentose nucleic acids from microbial sources*

Source of nucleic acid	No. of preparations analysed	Method of hydrolysis	Molar proportions calculated to total 4·00				Purines/ pyrimidines (mol. proportions)
			Adenine	Guanine	Cytosine	Thymine	
Bacteria:							
Tubercle bacilli							
Human strain	1	H.COOH	0·72	1·14	1·34	0·80	0·87
Bovine strain	1	H.COOH	0·71	1·17	1·35	0·76	0·89
Escherichia coli (mutant B/r):							
Synthetic medium	1	$HClO_4$	0·90	0·98	1·03	1·09	0·89
Broth	2	$HClO_4$	0·93	0·94	1·02	1·10	0·88
Viruses:							
E. coli phages							
T_2 synthetic medium	1	$HClO_4$	1·36	0·85	0·43	1·37	1·23
T_5 broth	1	H.COOH	1·34	0·82	0·41	1·44	1·17
T_2 broth	2	{H.COOH / $HClO_4$} *	1·33	0·73	0·505	1·44	1·08
Gipsy-moth polyhedral virus	4	{$HClO_4$ / H.COOH} *	0·86 ±0·01	1·20 ±0·01	1·12 ±0·04	0·81 ±0·04	1·07

* Mean results given.

The DNA preparations from *Escherichia coli* grown on broth and on the synthetic medium do not differ significantly in composition. The same is true of DNA isolated from bacteriophage T_5 from bacteria grown on the two media. Similarly, the differences between the human and bovine strains of tubercle bacilli may probably be accounted for by systematic error, although from the few analyses that have been done, the possibility of small real differences cannot be excluded. The differences between T_2 and T_5, on the other hand, we consider too great to be accounted for by analytical error, and represent a real difference between the compositions of the two nucleic acids.

The T_5 bacteriophage preparation isolated from bacteria grown on broth was also examined for the presence of RNA. A suspension of the virus containing 0·9 mg. nitrogen was precipitated with acetic acid at pH 4, dried, and incubated with N-sodium hydroxide at 37° for 12 hr. in order to convert any RNA to mononucleotides. The solution was made acid with hydrochloric acid and the precipitate containing DNA was removed on the centrifuge. The supernatant liquid was concentrated, placed on a chromatogram, and run with the *iso*propanol-hydrochloric acid solvent. In this solvent the ribonucleotides from RNA give two spots, one containing uridylic acid and the other a mixture of adenylic, guanylic and cytidylic acids. Both these run clear of the purines adenine and guanine. No well defined spots were distinguishable in these positions from the alkaline hydrolysate of bacteriophage; the amount of non-specific material present would have allowed the detection of RNA if present in an amount equivalent to more than 1 % of the DNA content of the virus.

Chromatograms of the gipsy-moth virus hydrolysed whole, without isolation of its nucleic acid, showed no uracil, thus confirming the absence of RNA from this virus. A polynucleotide integrated from the found amounts of the DNA bases would be equivalent to 15·2 % by weight of the virus, and account for 88 % of the total virus phosphorus. Since similar purine-pyrimidine analyses of purified DNA's have usually succeeded in accounting for only about 90 % of nucleic acid phosphorus (Wyatt, 1951*b*), it is probable that almost all of the virus phosphorus is included in its DNA. In one preparation, DNA was estimated by the diphenylamine reaction (Dische, 1930), which indicated the presence of 16·6 % DNA after a downward correction of 4 % had been applied to allow for the different purine/pyrimidine ratios in virus DNA and the thymus DNA standard (see Discussion).

DISCUSSION

Although the deoxypentose nucleic acids of only a small number of bacteria and viruses have been examined, these included organisms representative of several unrelated groups, and some generalizations are possible. Whereas DNA from a number of animals and at least one higher plant has a common pattern of composition, with more adenine and thymine than guanine and cytosine, a relatively small content of 5-methylcytosine, and a purine/pyrimidine ratio very close to unity (Wyatt, 1951*b*), this pattern is not shared by the nucleic acids of microbial origin. These are marked by the absence of 5-methylcytosine, by wide diversity in the relative proportions of the other bases, and by considerable

variation in the purine/pyrimidine ratio. There is some evidence that the composition of DNA varies less between related than between unrelated organisms, but many more nucleic acids will have to be analysed before we can state how closely these variations may parallel phylogenetic relationship.

Chargaff *et al.* (1950) have observed that DNA's from avian tubercle bacilli, *Serratia marcescens* and *Bacillus Schatz*, unlike those of higher animals, all contain an excess of guanine and cytosine over adenine and thymine, and on the strength of this they have proposed that DNA's may be divided into two main classes, the 'AT type' characteristic of animals and the 'GC type' of bacteria. According to this system, the bacteriophages would have the AT type and the gipsy-moth virus the GC type; *E. coli*, however, would have to be classified as 'transitional'. Again, only a more extensive survey of DNA composition can show whether this apparent grouping is more than fortuitous.

The variations in purine/pyrimidine ratio, whatever biological significance they may have, are of considerable practical importance, for the diphenylamine reaction commonly used for estimating DNA depends on the colour produced by purine-bound deoxypentose (Dische, 1930). If thymus nucleic acid, with a purine/pyrimidine ratio very close to 1·00, is used as the reference standard, estimations of DNA having a different purine/pyrimidine ratio will require correction by a corresponding factor. For example, estimations of DNA from phage T_2 will require downward correction by 3%—not, in this case, a very large error, but one which may possibly account for the recent finding by Cohen & Arbogast (1950) of enough DNA, estimated with diphenylamine, in phage T_2 to account for up to 105% of the total phosphorus of the virus. The error with phage T_5 or with *E. coli* would be greater still.

The compositions of DNA from host and bacteriophage are strikingly different and it is apparent that the bacterial nucleic acid could not be transferred directly in virus synthesis. This accords with the observations of Cohen (1948) and of Kozloff & Putnam (1950) that about 70–80% of the virus phosphorus is derived from inorganic phosphorus taken up from the medium after infection of the host. Kozloff & Putnam, however, consider that the remaining 20–30% of virus phosphorus comes from the bacterial DNA although this transfer may proceed via nucleotides.

SUMMARY

1. Deoxypentose nucleic acids have been isolated from the human and bovine strains of *Mycobacterium tuberculosis*, *Escherichia coli* (strain B/r), the bacteriophages T_2 and T_5 and the gipsy-moth polyhedral virus. Using paper chromatography the purine and pyrimidine compositions of these nucleic acids have been determined quantitatively.

2. Adenine, guanine, cytosine and thymine are present in all the nucleic acids, but 5-methylcytosine could not be detected.

3. The nucleic acids from different micro-organisms show a wide range of nucleotide composition differing from the general structural pattern found among the deoxypentose nucleic acids of higher organisms.

4. The compositions of deoxypentose nucleic acids from the bacteriophages T_2 and T_5 are distinct, although similar, but differ greatly from that of the host (*Escherichia coli*).

We wish to express thanks to the Wellcome Research Laboratories for the gift of dried tubercle bacilli, and to Prof. J. Komárek, Charles University, Prague, who facilitated the collection of diseased gipsy-moth caterpillars as a source of polyhedra. One of us (G. R. W.) was engaged on this project while on transfer of research from the Division of Entomology, Department of Agriculture, Ottawa, Canada.

REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Bergold, G. (1947). *Z. Naturforsch.* **2***b*, 122.
Bergold, G. (1950). *Viruses* 1950, ed. M. Delbruck, p. 60. California Institute Technology, Pasadena.
Bergold, G. & Pister, L. (1948). *Z. Naturforsch.* **3***b*, 406.
Chargaff, E. (1950). *Experientia*, **6**, 201.
Chargaff, E., Zamenhof, S., Brawerman, S. & Kerin, L. (1950). *J. Amer. chem. Soc.* **72**, 3825.
Cohen, S. S. (1948). *J. biol. Chem.* **174**, 295.
Cohen, S. S. & Arbogast, R. (1950). *J. exp. Med.* **91**, 607.
Demerec, M. & Fano, U. (1945). *Genetics*, **30**, 119.
Dische, Z. (1930). *Mikrochemie*, **8**, 4.
Hoagland, C. L., Lavin, G. I., Smadel, J. E. & Rivers, T. M. (1940). *J. exp. Med.* **72**, 139.
Johnson, T. B. & Coghill, R. D. (1925). *J. Amer. chem. Soc.* **47**, 2838.
Kozloff, L. M. & Putnam, F. W. (1950). *J. biol. Chem.* **182**, 229.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
Mirsky, A. E. & Pollister, A. W. (1946). *J. gen. Physiol.* **30**, 117.
Putnam, F. W. (1950). *Science*, **111**, 481.
Putnam, F. W., Kozloff, L. M. & Neil, J. C. (1949). *J. biol. Chem.* **179**, 303.
Robinow, C. F. (1945). Addendum to *The Bacterial Cell*, p. 353. By Dubos, R. J. Cambridge, Mass.: Harvard University Press.
Schmidt, G. & Thannhauser, S. J. (1945). *J. biol. Chem.* **161**, 83.
Sevag, M. G., Lackman, D. B. & Smolens, J. (1938). *J. biol. Chem.* **124**, 425.
Vischer, E., Zamenhof, S. & Chargaff, E. (1949). *J. biol. Chem.* **177**, 429.
Witkin, E. M. (1946). *Proc. nat. Acad. Sci., Wash.*, **32**, 59.
Wyatt, G. R. (1950). *Nature, Lond.*, **166**, 237.
Wyatt, G. R. (1951*a*). *Biochem. J.* (in the Press).
Wyatt, G. R. (1951*b*). *Biochem. J.* (in the Press).

The Metabolism of Nitrate and Nitrite in the Sheep

2. HYDROGEN DONATORS IN NITRATE REDUCTION BY RUMEN MICRO-ORGANISMS *IN VITRO*

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Sapiro, Hoflund, Clark & Quin (1949) demonstrated the reduction of nitrate to nitrite by rumen contents *in vitro*, and Lewis (1951) has shown that nitrate is reduced to nitrite and ammonia *in vivo* in the rumen of the sheep. Since many bacteria are known to reduce nitrate to nitrite and ammonia it is reasonable to assume that the appearance of these compounds in the rumen of the sheep after dosing with nitrate is the result of the reducing activity of the rumen micro-organisms.

The reduction of nitrate by pure cultures of bacteria has been studied by a number of workers: Quastel & Stephenson (1925) and Quastel, Stephenson & Whetham (1925) showed that *Escherichia coli* reduces nitrate to nitrite in the presence of such hydrogen donators (H-donators) as lactate, glycerol, succinic acid or malic acid, and Stickland (1931), using washed suspensions, showed that the process was catalysed by an enzyme activating nitrate (nitratase). Aubel (1938) studied the reduction of nitrate to ammonia by *Esch. coli*, with glucose as H-donator. Stephenson & Stickland (1931) demonstrated that organisms possessing both hydrogenase and nitratase reduced nitrate quantitatively to nitrite in the presence of hydrogen. In contrast to these results, Woods (1938) showed that two strains of *Esch. coli* and one strain of *Clostridium welchii* were able, in the presence of hydrogen, to reduce nitrate quantitatively to ammonia. In this reduction there was a transient appearance of nitrite, and Woods provided evidence that both nitrite and hydroxylamine were intermediates in the reaction.

The H-donators in the reduction of nitrate in the rumen are unknown. It is not possible to make a detailed analysis of this process in the rumen of the sheep because of the complexity of the reactions occurring therein. Since the reduction proceeds rapidly even in an animal that has been fasted for 16 hr. it would be difficult to produce any significant change in the rate or extent of the nitrate reduction by the introduction of a potential H-donator into the rumen. However, using washed micro-organisms prepared according to the method of Elsden & Sijpesteijn (1950*a*), it has been possible to study the process under controlled conditions. Suspensions of rumen bacteria prepared in this way reduce nitrate to nitrite and ammonia, in the presence of a number of H-donators, and of those tested hydrogen was the most active.

* Seconded from the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge.

EXPERIMENTAL METHODS

Washed cell suspensions. A volume of rumen fluid was withdrawn from the rumen of a sheep fitted with a permanent rumen fistula (Phillipson & Innes, 1939). It was filtered through muslin and the filtrate spun at approx. 1000 rev./min. for 1 min. on the Measuring and Scientific Equipment Ltd. (M.S.E.) minor centrifuge. The deposit was discarded and the supernatant centrifuged at approx. 3000 rev./min. for 30 min. The residue was suspended in 0·05 M phosphate buffer pH 6·5 which had been previously boiled and to which 50% (w/v) $Na_2S.9H_2O$ had been added to a final concentration of 0·02% (w/v). This suspension was again spun at 3000 rev./min. for 30 min., and the residue resuspended in the de-oxygenated buffer.

Manometric methods. The reactions were carried out in Warburg manometer vessels at 37°. The main compartments of the vessels contained 2 ml. of the washed cell suspension in the de-oxygenated buffer, and after thermal equilibrium had been attained the potential H-donator and the nitrate were added from the side bulb. The centre wells contained 0·2 ml. 20% (w/v) KOH, to absorb any CO_2 produced.

At the end of the incubation period of 2 hr. unless otherwise stated, samples of the reaction fluids were analysed for both nitrite and ammonia. When gaseous H_2 was studied as H-donator two series of controls were carried out. The blank NH_3 production was determined when the suspension was incubated in the presence of nitrate but with N_2 as the gas phase, and the blank H_2 consumption was obtained in the absence of nitrate with H_2 as the gas phase. The N_2 was freed from O_2 by passing over Cu turnings heated to 400°. All values of NH_3 formation have been corrected for the small initial NH_3 content of the washed cell suspensions.

Ammonia was estimated on 1 ml. samples by the method of Conway & O'Malley (1942). *Nitrite* was determined by the Griess-Ilosvay method (Association of Official Agricultural Chemists Handbook, 1947): 1 ml. samples from the manometer vessels were clarified by the addition of 3 ml. lead acetate (saturated aqueous solution, diluted 1 in 10), and 1 ml. saturated Na_3PO_4; the mixture was then shaken and filtered, and a sample taken for the colorimetric estimation.

Total nitrogen was determined by the Kjeldahl method as modified by Chibnall, Rees & Williams (1943), and the NH_3 trapped in the boric acid reagent of Conway & O'Malley (1942).

RESULTS

Gaseous hydrogen as a donator

Nitrate reduction. Gaseous hydrogen was first tested as a donator because there was some evidence that rumen micro-organisms activated hydrogen, and also because of the work of Stephenson & Stickland (1931) and Woods (1938) previously referred to. Table 1 shows that in the presence of hydrogen nitrate is reduced quantitatively to ammonia with the consumption of an equivalent amount of hydrogen, according to equation (1).

$$KNO_3 + 4H_2 \rightarrow NH_3 + 2H_2O + KOH. \quad (1)$$

The observed ammonia production (after the initial ammonia of the suspension had been subtracted) was corrected for the ammonia produced in the control experiments carried out in nitrogen. The uptake of hydrogen was corrected for the slight uptake which occurred in the absence of nitrate. After these corrections both the ammonia production and hydrogen uptake were equivalent to 95–100% of the theoretical values for the complete reduction of nitrate to ammonia.

Nitrite and hydroxylamine reduction. The reduction of hydroxylamine and nitrite by hydrogen was next studied (cf. Woods, 1938). For comparison, vessels were included which contained nitrate. The course of the hydrogen uptake in a typical experiment is shown in Fig. 1. The blank uptake of hydrogen by the suspension in the absence of substrate never exceeded 3% of the uptake in the presence of nitrate. Consumption of hydrogen in the presence of nitrate, nitrite and hydroxylamine corresponded to

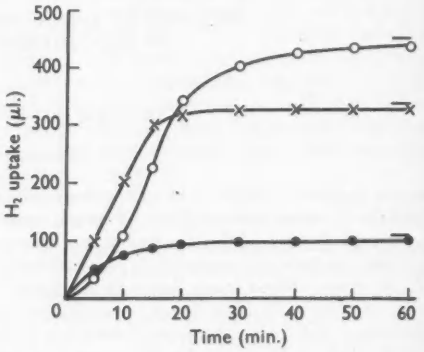

Fig. 1. Hydrogen uptake by washed micro-organisms in the presence of nitrate, nitrite or hydroxylamine. ○—○, NO_3; ×—×, NO_2; ●—●, NH_2OH; —, theoretical uptake for reduction to ammonia. Corrections have been applied for the hydrogen uptake in the absence of substrate. 2 ml. washed cell suspension in 0·05 M-phosphate buffer pH 6·5 (10·7 mg. total N/100 ml.). 5 µmol. substrate in 0·2 ml. water.

reduction to ammonia according to equations (1), (2) and (3).

$$NaNO_2 + 3H_2 \rightarrow NH_3 + NaOH + H_2O, \quad (2)$$

$$NH_2OH + H_2 \rightarrow NH_3 + H_2O. \quad (3)$$

Table 1. *Hydrogen uptake and ammonia formation by washed suspensions in the presence of nitrate*

(2 ml. washed cell suspension (9·7 mg. total N/100 ml.); 4 µmol. KNO_3. Theoretical values are for complete reduction of the KNO_3 to NH_3.)

		H_2 uptake		NH_3 production	
Substrate	Gas phase	(µl.)	Percentage theory (corr.)	(µmol.)	Percentage theory (corr.)
KNO_3	H_2	363	97	4·78	97
KNO_3	H_2	360	96	4·83	98
KNO_3	N_2	1	—	0·97	—
KNO_3	N_2	4	—	0·85	—
None	H_2	14	—	0·51	—
None	H_2	16	—	0·35	—

Table 2. *The reduction of nitrate, nitrite and hydroxylamine to ammonia by washed suspensions under hydrogen gas phase*

(2 ml. washed cell suspension (6·7 mg. total N/100 ml. suspension).)

					Molar ratios		
Substrate	(µmol.)	Gas phase	H_2 uptake (µmol.)	NH_3 formation (µmol.)	Substrate added	H_2 taken up (corr.)	NH_3 formed (corr.)
No substrate	—	H_2	0·4	0·4	—	—	—
NH_2OH	16	H_2	14·4	17·7	1	0·9	1·1
NH_2OH	16	N_2	—	0·9	—	—	—
$NaNO_2$	5	H_2	14·3	5·2	1	2·8	0·9
$NaNO_2$	5	N_2	—	0·8	—	—	—
KNO_3	4	H_2	15·6	5·0	1	3·8	1·0
KNO_3	4	N_2	—	0·9	—	—	—

At the completion of the experiments samples of the contents of the manometer vessels were immediately withdrawn for the estimation of nitrite and ammonia. The nitrite concentration accounted for less than 2% of added nitrate or nitrite. After applying the appropriate corrections the results in Table 2 show that nitrate, nitrite and hydroxylamine are all reduced to ammonia in good agreement with the stoicheiometric relationships of equations (1)–(3).

Effect of pH *on the reduction.* The effect of hydrogen ion concentration on the rates of these processes was investigated. The preparation of the washed cell suspension differed in that the rumen micro-organisms were both washed and suspended in a special saline which was prepared as follows: a stock solution of 0·9% (w/v) sodium chloride was boiled immediately before use and cooled rapidly under the tap; to this was added 50% sodium sulphide ($Na_2S.9H_2O$) to a final concentration of 0·02% (w/v) and sufficient N-hydrochloric acid to bring the pH to 6·5. The main compartment of the manometer vessels contained 1 ml. of the suspension and 1 ml. of 0·2M-phosphate buffer. The buffers covered the range pH 5·4–7·3. A control was set up at each pH containing no substrate. The nitrate, nitrite or hydroxylamine (0·2 ml., 0·05M) was tipped in from the side arm after thermal equilibrium had been attained. Initial rates of hydrogen uptake, from 3 to 10 min. after tipping in the substrate, were determined and each corrected for the small gas uptake in the controls. The results are presented in Fig. 2. The pH optima for nitrate and nitrite reduction are sharp and at 6·2 and 5·6 respectively, whereas with hydroxylamine there is a broad plateau over the range of 6–7. The optima for the analogous processes catalysed by *Cl. welchii* were 7·5, 6·7 and above 8 respectively (Woods, 1938), i.e. occupying similar relative positions. These findings are of interest in view of the fact that the normal pH of the rumen is around 6·5.

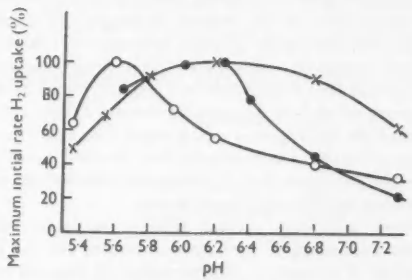

Fig. 2. pH activity curves for the reduction of nitrate, nitrite and hydroxylamine by the washed suspensions in an atmosphere of hydrogen. ●—●, NO_3; ○—○, NO_2; ×—×, NH_2OH. Suspension contained 33·2 mg. total N/100 ml.

Detection of intermediates. These results, like those of Woods (1938), suggest that nitrite and hydroxylamine are intermediates in the reaction, and an attempt was made to ascertain whether nitrite accumulates at any stage during the reduction of nitrate. Nitrite is known to accumulate *in vivo* (Lewis, 1951) and in the reaction catalysed by washed suspension of *Cl. welchii* (Woods, 1938). Nitrate was incubated with the washed cell suspension of rumen bacteria in an atmosphere of hydrogen, and the concentrations of nitrite and ammonia were determined at intervals during the experiment. The reaction was complete in 60 min. For convenience a series of manometers was used, and the reactions were stopped individually when a suitable hydrogen uptake had been recorded. The results are presented in Fig. 3. The total hydrogen uptake and ammonia production again corresponded to complete reduction of the nitrate to ammonia. However, during the first 20 min. there was an accumulation of nitrite. This observation agrees with the *in vivo* findings that when nitrate is introduced into the rumen of the sheep there is a rapid but transient appearance of nitrite (Lewis, 1951).

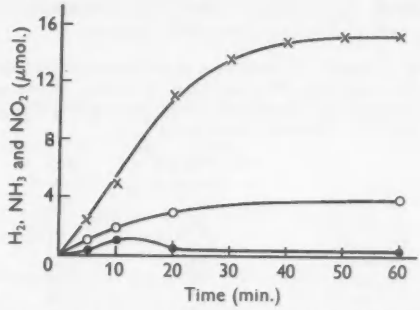

Fig. 3. Hydrogen uptake, ammonia formation and nitrite appearance and disappearance when washed cell suspensions are incubated with nitrate in the presence of hydrogen. ×—×, μmol. H_2 uptake; ○—○, μmol. NH_3 formation; ●—●, μmol. NO_2 formation. 2 ml. washed suspension in 0·05M-phosphate buffer pH 6·2. (18·8 mg. total N/100 ml.) 4 μmol. KNO_3.

Hydrogen donators other than gaseous hydrogen

Other potential hydrogen donators tested for nitrate reduction by the rumen micro-organisms included substances known to be present in the rumen, e.g. the lower fatty acids, and others possibly present under certain conditions, for example lactate, succinate and glucose. The reactions were carried out in manometer vessels in nitrogen. The substrates were incubated for 2 hr.

with washed rumen bacteria in the presence of nitrate. The ability of the compounds to act as donators was assessed by the amounts of nitrite and ammonia produced over and above that observed in a control without donators. It was found that the fatty acids, acetic, propionic, and *n*-butyric, were completely inactive as hydrogen donators, but succinate or formate gave a considerable production of nitrite and ammonia.

A more comprehensive experiment was carried out to determine the relative efficiency of various substances as hydrogen donators for nitrate reduction. A typical series of results is shown in Table 3. The

Table 3. *The production of nitrite and ammonia from nitrate by washed rumen micro-organisms in presence of various potential* H*-donators*

(2 ml. washed cell suspension in 0·05 M-phosphate buffer pH 6·2 containing 20·3 mg. total N/100 ml. Hydrogen donator (0·4 ml., 0·1 M) and 0·2 ml. 0·1 M-KNO_3 (280 μg. nitrate N) in side bulb. Incubation 2 hr.)

H-donator	Ammonia N formed (μg.)	Nitrite N formed (μg.)
Control	26	3
Hydrogen	174	21
Succinate	94	18
Formate	97	12
DL-Lactate	77	16
Citrate	74	11
D-Glucose	62	22
L(−)-Malate	64	17
D-Mannitol	63	5
Glycerol	52	3
D-Xylose	44	11
Ethanol	32	5

substrates were incubated for 2 hr. with the washed cells in the presence of nitrate. The reduction was not complete in any instance, but the use of more cells led to a proportional increase in the blank production of nitrite and ammonia, whereas if the incubation time were increased the biochemical activity of the suspension would probably undergo considerable change. It will be considered that there was a significant reduction of the nitrate when the production of nitrite and ammonia in the presence of a hydrogen donator was at least double that in the control. On this basis there was a significant reduction of nitrate in the presence of hydrogen, succinate, formate, DL-lactate, citrate, D-glucose, L(−)-malate, and D-mannitol, but not in the presence of glycerol, D-xylose or ethanol.

DISCUSSION

Of the compounds tested as possible hydrogen donators for the reduction of nitrate by rumen micro-organisms, only the lower fatty acids are definitely known to occur in the rumen under the conditions of the *in vivo* investigations (Lewis, 1951; Elsden, 1945): these were, however, totally inactive. Succinate is probably an intermediate in cellulose breakdown (Sijpesteijn, 1949). Lactate and glucose may occur in the rumen under certain conditions (Elsden, 1945; Phillipson, 1942), and Sijpesteijn (1950) has obtained evidence that formic acid is possibly a product of cellulose decomposition by *Ruminococcus flavifaciens*, a cellulose-decomposing organism isolated from the rumen of both sheep and cattle. It is not known whether the other compounds tested as donators occur in the rumen.

Hydrogen, the most active of the substances tested, is known to be a common end product of many bacterial fermentations, and *Veillonella gazogenes*, isolated by Johns (1949) from the rumen of the sheep, is an active hydrogen producer. Elsden & Sijpesteijn (1950*b*) have shown that the rumen organisms possess an active hydrogenase, and a large uptake of hydrogen has been recorded in the presence of carbon dioxide. However, Lugg (1938) states that hydrogen is not a constituent of rumen gases. The situation would be clarified were it possible to show that hydrogen is utilized in the rumen as rapidly as it is produced.

Thus, although several substances have been shown to be active hydrogen donators for the reduction of nitrate in the presence of washed cell suspensions of rumen micro-organisms, it is not as yet possible to state definitely that these account for the process *in vivo*. The transitory appearance of nitrite in the early stages following the addition of nitrate is found both *in vivo* and *in vitro*.

SUMMARY

1. Hydrogen is a very active donator for the reduction of nitrate, nitrite and hydroxylamine to ammonia, in the presence of washed micro-organisms from sheep rumen.

2. Nitrite is an intermediary in the reduction of nitrate, and appears transitorily in the early stages of reduction by hydrogen.

3. The pH optima of nitrate and nitrite reduction in an atmosphere of hydrogen are 6·5 and 5·6 respectively, whereas the optimum for hydroxylamine reduction shows a broad plateau over the range of pH 6–7.

4. Formate, succinate, lactate, citrate, glucose, malate and mannitol are hydrogen donators for nitrate reduction, but at lower rates than hydrogen. Glycerol, xylose and ethanol have little activity and acetate, propionate and *n*-butyrate are inactive.

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REFERENCES

Association of Official Agricultural Chemists (1947). *Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists*, 2nd ed. Washington.
Aubel, E. (1938). *C.R. Soc. Biol., Paris*, **128**, 45.
Chibnall, A. C., Rees, H. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Conway, E. J. & O'Malley, E. (1942). *Biochem. J.* **36**, 655.
Elsden, S. R. (1945). *J. exp. Biol.* **22**, 51.
Elsden, S. R. & Sijpesteijn, A. K. (1950*a*). *J. gen. Microbiol.* **4**, xi.
Elsden, S. R. & Sijpesteijn, A. K. (1950*b*). Personal communication.
Johns, A. T. (1949). *Nature, Lond.*, **164**, 620.
Lewis, D. (1951). *Biochem. J.* **48**, 175.
Lugg, J. W. H. (1938). *J. agric. Sci.* **28**, 688.
Phillipson, A. T. (1942). *J. exp. biol.* **19**, 186.
Phillipson, A. T. & Innes, J. R. M. (1939). *Quart. J. exp. Physiol.* **29**, 333.
Quastel, J. H. & Stephenson, M. (1925). *Biochem. J.* **19**, 660.
Quastel, J. H., Stephenson, M. & Whetham, M. D. (1925). *Biochem. J.* **19**, 304.
Sapiro, M. L., Hoflund, S., Clark, R. & Quin, J. I. (1949). *Onderstepoort J. vet. Sci.* **22**, 357.
Sijpesteijn, A. K. (1949). *Antonie van Leeuwenhoek*, **15**, 49.
Sijpesteijn, A. K. (1950). Private communication.
Stephenson, M. & Stickland, L. H. (1931). *Biochem. J.* **25**, 205.
Stickland, L. H. (1931). *Biochem. J.* **25**, 1543.
Woods, D. D. (1938). *Biochem. J.* **32**, 2000.

Displacement Chromatography on Synthetic Ion-exchange Resins

7. SEPARATIONS USING A STRONGLY BASIC RESIN

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Previous communications (Partridge, 1949, 1950) have reported the use of an ion-exchange resin for the separation of amino-acids from a protein hydrolysate into eight groups, each consisting of no more than three amino-acids. The separation was carried out as a displacement chromatogram, using a strongly acidic cation-exchange resin, and displacing the mixture with ammonia or sodium hydroxide solution. A fractionation of this kind forms a logical starting point for a scheme aiming at the complete separation and isolation of all the component amino-acids in the complex mixture; and the preliminary group separation has thus been termed the 'primary fractionation'. When carried out with a multiple column having a top section packed with sulphonated polystyrene resin (Partridge, Brimley & Pepper, 1950) the primary fractionation results in the immediate isolation of three amino-acids in a pure condition; these are aspartic acid, lysine and arginine. The other amino-acids appear as mixed bands as follows: band II, glutamic acid, serine and threonine; band III, glycine and alanine; band IV, valine and proline; band V, methionine, leucine and isoleucine; band VI, histidine and the leucines.

Various procedures have been described for the further separation of the components of some of these bands, e.g. the separation of glutamic acid from band II by the use of a weakly basic resin (Partridge & Brimley, 1949) or the separation of methionine from leucine by the use of a cation-exchange resin at higher temperatures (Partridge & Brimley, 1951). However, the appearance in commercial production of several new strongly basic resins now offers the possibility of separating other mixed bands and also of bringing all the secondary separations within a generalized scheme.

The separation of a series of amino-acids in displacement chromatograms using strongly acidic resins is determined largely by the order of the values of pK_1 (COOH) for the members of the series (Davies, 1949). Similarly, the order of displacement from strongly basic anion-exchangers is largely determined by the order of pK_2 (NH_3^+) (Davies, Hughes & Partridge, 1950) and thus displacement from an anion-exchanger should give rise, not to a simple reversal of the order found with cation-exchangers, but rather to an entirely new grouping of the amino-acids. The work described in this paper shows that this prediction is borne out in practice and that use may be made of the differences for securing further separations.

EXPERIMENTS AND RESULTS

The ion exchanger used for these experiments was Dowex 2 (obtainable from the Dow Chemical Company, Midland, Michigan). The resin is a cross-

linked polymer containing strongly basic quaternary groups. It is a cream-coloured material, and the sample used was in the form of micro-beads 250–500 mesh/in. The resin was supplied in the chloride form, and was regenerated before use with carbonate-free 2N-sodium hydroxide solution until chloride-free. (It was found convenient to prepare carbon dioxide-free alkali by adding 10 g. barium hydroxide crystals to each l. of 2N-NaOH solution, the solution being filtered immediately before use). After regeneration the resin was washed with carbonate-free distilled water until the alkalinity of the eluate fell below pH 8. Carbonate-free solutions were used at all stages, since it was found that if any carbonate became adsorbed on the resin, carbon dioxide was released by the acid used as displacement developer, and this seriously disturbed the chromatogram.

The preliminary characterization of the resin has already been reported (Davies *et al.* 1950), the results showing that the widths of the boundaries (as defined by Partridge & Westall, 1949) for hydrochloric acid were 1–1·5 cm. when the rate of progression of the boundaries was in the range 2–4 cm./hr. The boundaries were considerably wider than those observed with the cation exchanger Zeo-Karb 215 at comparable rates of flow, and for this reason, in separation experiments with Dowex 2, the flow rate was adjusted to maintain a rate of progression of the boundaries below 3 cm./hr. The resin behaved unifunctionally for strong acids, its capacity being 3·7 m-equiv./g. dry resin.

Construction of the column

The column was prepared in two sections connected in series and mounted one above the other. The upper section consisted of a filtration tube of diameter 1·6 cm. and height 15 cm. It was packed with 10 g. (air-dry wt.) of Dowex 2 (water content, 16·0%), the height occupied by the resin being 11·5 cm. The lower filtration tube was 1·0 cm. in diameter and 11 cm. in height. It contained 3 g. of resin of the same particle size as that used for the upper section (height occupied by the resin, 8·4 cm.). The general arrangement of the assembly was that described by Partridge & Brimley (1951). Before use the column was treated alternately with 0·2N-HCl and CO_2-free 2N-NaOH in order to remove impurities and compact the resin bed. The column was finally regenerated and washed as described above.

Separation experiments

The plan adopted for these experiments was to carry out a standard displacement procedure with a series of known amino-acid mixtures representing the mixed bands obtained in the primary fractionation. The amount of amino-acid mixture was approximately 12 mmol. in each case. The mixture was dissolved in water (70 ml.) and boiled to expel carbon dioxide. After cooling the solution was applied to the column at a rate of 40–50 ml./hr. and the column then washed with a little carbon dioxide-free water. The displacement developer (0·1N-hydrochloric acid, 650 ml.) was then applied at the same rate. The effluent was collected in fractions by an automatic device which changed the receiver at pre-set intervals of time. The column was usually allowed to run unattended overnight, and the following morning one drop was taken from each receiver for the preparation of a filter-paper chromatogram. The results of the qualitative analyses so obtained are shown in Fig. 1.

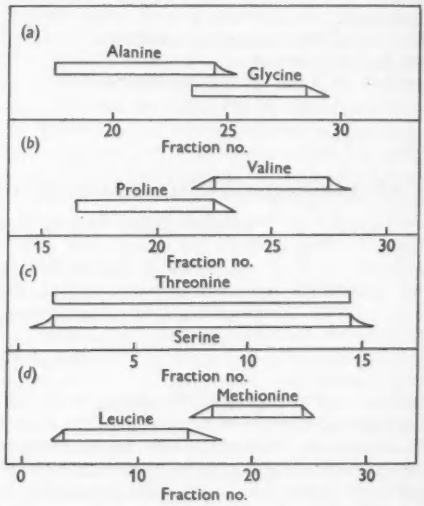

Fig. 1. Chromatograms prepared by displacing mixtures of (*a*) alanine and glycine, (*b*) proline and valine, (*c*) serine and threonine, (*d*) leucine and methionine from a column packed with Dowex 2. Displacement developer, 0·1N-HCl.

Fig. 1 (*a*), (*b*) and (*d*) shows that excellent separations were obtained with mixtures of alanine and glycine, proline and valine, and leucine and methionine respectively; however, serine and threonine showed no tendency to separate.

The isolation of L-*alanine and glycine from the hydrolysis products of egg albumin*

The primary fractionation of an acid-hydrolysate of commercial egg albumin has already been described (Partridge, 1949). Since, at the time this fractionation was carried out, no satisfactory procedure was available for the separation of glycine from alanine, the two amino-acids (comprising band III) had been collected as a crystalline mixture for storage. This mixture was used for the present experiments.

The column used in the fractionation experiments described above was adapted for a larger output by introducing a third and larger section. This was mounted on top of the existing assembly and was 2·1 cm. in diameter and 22 cm.

in height. It was packed with Dowex 2 in bead form, wet screen analysis 100–200 mesh/in., the resin occupying a height of 17·7 cm. in the filtration tube. The mixture of glycine and alanine (4·0 g.) was dissolved in water (100 ml.), brought to the boil to expel CO_2 and cooled. It was applied to the column at a rate of 30 ml./hr. and was followed by the displacement developer (0·15 N-HCl) which was applied at the same rate. The fraction collector was set to change the receiver every 20 min. After completion of the experiment a qualitative analysis of the contents of each receiver was carried out by means of paper chromatography. The first twenty-one fractions contained alanine only. This was followed by two fractions which contained a mixture of glycine and alanine, and which were rejected. The following ten fractions contained glycine only, while the 34th fraction also contained glycine, but was contaminated with HCl and was rejected.

Recovery of L-*alanine.* The solution containing L-alanine was concentrated under reduced pressure to the point at which crystallization began. Two volumes of hot ethanol were then added to the warm solution. On cooling, L-alanine crystallized in fine needles, wt. 2·54 g. Found: N 15·7. Calc. for $C_3H_7O_2N$: N, 15·7 %; $[\alpha]_D^{20°} + 14·5°$ in 5·9 N-HCl (*c*, 3·37, 2 dm.). The yield represented 3·44 % of the dry weight of protein taken.

Recovery of glycine. The glycine solution was concentrated under reduced pressure to a colourless viscous liquid. About one-half the volume of ethanol was then added, and on scratching the glycine crystallized in small prisms, wt. 1·07 g. Found: N, 18·4. Calc. for $C_2H_5O_2N$: N, 18·7 %. The yield represented 1·44 % of the dry weight of protein taken.

DISCUSSION

The mechanisms which control the separation of a mixture of amino-acids in a displacement chromatogram have been discussed by Davies (1949). Considering the case of displacement from a column of strongly acidic resin, Davies pointed out that separation would be expected to depend upon: (*a*) the extent of ionization of the individual amino-acids in their passage down the column, (*b*) the charges carried by the ions present, and (*c*) the van der Waals forces between the resin and the various species present. It was further shown that, for a cation-exchange column, mechanism (*a*) would result in an order of displacement for a series of amino-acids which would be qualitatively identical with the order of dissociation constants for the process $A^+ \rightleftharpoons H^+ + A^\pm$; that is to say, the order of pK_1 (COOH) for monoamino-acids. The operation of mechanism (*b*) would be expected to result in a retardation of the diamino-acids since they are able to form bivalent cations. Similarly, the operation of mechanism (*c*) should result in a greater relative retardation of those amino-acids of higher molecular weight, or those possessing a specific structure such as the aromatic nucleus which would confer a higher adsorptive affinity towards the resin.

In the case of a displacement chromatogram carried out with a strongly basic anion-exchanger, mechanism (*a*) becomes dependent upon the dissociation constant for the reaction $A^\pm \rightleftharpoons H^+ + A^-$; that is to say, upon the values of pK_2 (NH_3^+) for monocarboxylic amino-acids; and in this case amino-acids forming bivalent anions should be strongly retarded (mechanism *b*). The effect of van der Waals forces should operate in the same sense in both cases; amino-acids associated with a large van der Waals contribution being retarded with both types of resin.

The recent introduction of resins with strongly basic properties has enabled this hypothesis to be tested by a direct comparison of the order of displacement of the same set of amino-acids with resins of both types. The order of displacement of a series of amino-acids from Dowex 2 was determined by direct experiment. A mixture of fourteen amino-acids was displaced from a column with 0·1 N-hydrochloric acid and the effluent from the column analysed by paper chromatography. The result is given in Table 1 (column 4) which includes, for comparison, values of pK_2 (NH_3^+) taken from Cohn & Edsall (1943). Arginine was only slightly retarded by the resin, and appeared as an 'elution peak' in front of the main displacement pattern. The other amino-acids followed in approximately the expected order, but proline, histidine, methionine and phenylalanine appeared rather lower in the order than would be expected from their dissociation constants. The amino-acids included in the brackets formed mixed bands, and as will be seen from the table, three amino-acids, valine, leucine and glycine, which form a mixed band, have almost identical values of pK_2.

Table 1 (column 1) also includes the order of displacement (Partridge, 1949) of the amino-acids from Zeo-Karb 215, a strongly acidic resin. The order is approximately that expected from the values of pK_1, but here again proline, methionine and phenylalanine are relatively retarded. A high contribution from van der Waals forces would be expected for phenylalanine and to a lesser extent for methionine since these amino-acids are strongly adsorbed by charcoal (Tiselius, 1947). Proline is not strongly adsorbed by charcoal, however, and this may be taken to suggest that a further mechanism leading to retardation may be operative.

Column 2 (Table 1) lists the order of displacement of the amino-acids from Zeo-Karb 215 at a higher temperature. It will be seen that the order is somewhat different from that at 20°, and that the behaviour of proline and methionine more nearly approaches that expected from the values of pK_1. This indicates that the effect of the van der Waals contribution for these amino-acids is relatively less important at 60° than at 20°. Use has been made of the change in order to secure the isolation of valine, proline, methionine and leucine from the hydrolysis products of a protein (Partridge & Brimley, 1951).

Table 1. *Order in which amino-acids appear in the the effluent from columns packed with Zeo-Karb 215, sulphonated polystyrene or Dowex 2*

(The brackets include groups of amino-acids which form mixed bands. Values of pK_1 and pK_2 are included for comparison. * marks amino-acids occupying anomalous positions in the order of displacement.)

Column no. (1)	(2)	(3)		(4)	
Zeo-Karb 215 at 20°	Zeo-Karb 215 at 60°	Sulphonated polystyrene at 20°		Dowex 2 at 20°	
Order of displacement	Order of displacement	Order of displacement	pK_1 (COOH)	Order of displacement	pK_2 (NH_3^+)
Aspartic acid	Aspartic acid	Aspartic acid	1·88	Arginine	12·48 (Guanidine)
Glutamic acid	Glutamic acid	Threonine	—	Lysine	10·53 (ϵNH_3^+)
Serine, Threonine	Serine, Threonine	Serine, Glutamic acid	2·21, 2·19	Proline*	10·60
Glycine, Alanine	Glycine, Alanine	Proline*	1·99	Alanine	9·69
Valine	Proline*	Glycine, Alanine, Valine	2·34, 2·34, 2·32	Valine, Leucine, Glycine	9·62, 9·60, 9·60
Proline*	Valine	Threonine	—	Threonine	—
Methionine*, Leucine	Methionine*, Leucine	Serine, Methionine*, Leucine	2·28, 2·36	Serine	9·15
Phenylalanine*	Phenylalanine*	Phenylalanine*	1·83	Histidine*	9·17
Bivalent cations	Bivalent cations	Bivalent cations	—	Methionine*	9·21
				Bivalent anions	—
				Phenylalanine*	9·13

The order of displacement of the amino-acids from a sulphonated polystyrene resin is given in column 3. This order is similar to that shown by Zeo-Karb 215 except in the case of proline, which now appears in front of the glycine-alanine band instead of behind it. In addition there is a small shift forward in the position of threonine. The remarkable difference in the behaviour of proline with the two resins throws light on the extremely anomalous position occupied by proline in the order of displacement from Zeo-Karb 215 at room temperature. As has already been mentioned, proline is not strongly adsorbed by charcoal, but it appears from these results that molecular adsorption may be important nevertheless. Zeo-Karb 215 is a nuclear sulphonic acid resin based on phenol-formaldehyde and thus contains phenolic hydroxyl groups in addition to sulphonic acid residues. The sulphonated polystyrene resin on the other hand contains no hydroxyl groups. This suggests that the anomalous adsorption of proline with Zeo-Karb 215 is due primarily to intermolecular forces that are specifically dependent upon the content of phenolic hydroxyl in the resin.

There may be further factors, however, which contribute towards the high affinity of proline. Proline is distinguished from the other α-amino-acids by ring closure on the α-amino group. This introduces a steric factor which may affect the coulombic forces between proline ions and the charged groups on the resin. Further, adsorption of an amino-acid by a strongly acidic or strongly basic resin would be expected to lead to a change in the degree of hydration of both the resin and the solute, and in certain cases this may contribute towards the stability of an ion-resin complex. Both these effects have been discussed by several workers in relation to the exchange of inorganic cations on strongly acidic resins (cf. Gregor, 1948; Cosgrove & Strickland, 1950).

The contribution of van der Waals forces for a series of acidic and basic dyes containing the aromatic nucleus has been discussed by Weiss (1950), who shows that such molecules may be adsorbed by ion-exchange resins when either the ion or the resin is uncharged, but the dye is eluted when both the ion and the resin have charges of the same sign. This type of adsorption is independent of the normal ion-exchange process and promises to be of value as a means of isolating the van der Waals contribution for independent study.

The separation experiments given in the text and the orders of displacement given in Table 1 show that it would be possible to effect an almost complete separation of the fourteen amino-acids listed, by use of two columns only. In this scheme, the primary fractionation would be carried out using a strongly acidic resin and the mixed bands so obtained would be separated by use of a strongly basic resin. The only exception to complete separation that would be expected is the leucine-isoleucine mixture, since this pair of amino-acids forms a mixed band on both types of resin. A simple scheme of this kind is being tested experimentally.

SUMMARY

1. The use of a strongly basic anion-exchange resin (Dowex 2) for the separation of amino-acids by displacement chromatography has been investigated.

2. Mixtures of the following pairs of amino-acids were separated: glycine-alanine, valine-proline, methionine-leucine. A mixture of serine and threonine did not separate.

3. The isolation of glycine and L-alanine from a protein hydrolysate is described.

4. The order of displacement of fourteen amino-acids was determined by displacing the mixture with dilute hydrochloric acid solution from a column of Dowex 2. This order was substantially the same as the order of pK_2 (NH_3^+) for the monocarboxylic amino-acids, and the result is discussed in relation to the theory of Davies (1949).

5. The use of a strongly basic resin in a simple scheme for the isolation of amino-acids from complex mixtures is discussed.

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REFERENCES

Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides*. New York: Reinhold.
Cosgrove, J. D. & Strickland, J. D. H. (1950). *J. chem. Soc.* p. 1845.
Davies, C. W. (1949). *Biochem. J.* **45**, 38.
Davies, C. W., Hughes, R. B. & Partridge, S. M. (1950). *J. chem. Soc.* p. 2285.
Gregor, H. P. (1948). *J. Amer. chem. Soc.* **70**, 1293.
Partridge, S. M. (1949). *Biochem. J.* **44**, 521.
Partridge, S. M. (1950). *Chem. & Ind.* **20**, p. 383.
Partridge, S. M. & Brimley, R. C. (1949). *Biochem. J.* **44**, 513.
Partridge, S. M. & Brimley, R. C. (1951). *Biochem. J.* **48**, 313.
Partridge, S. M., Brimley, R. C. & Pepper, K. W. (1950). *Biochem. J.* **46**, 334.
Partridge, S. M. & Westall, R. G. (1949). *Biochem. J.* **44**, 418.
Tiselius, A. (1947). *Advanc. protein Chem.* **3**, 67.
Weiss, D. E. (1950). *Nature, Lond.*, **166**, 66.

The Micro-estimation of Porphyrins by Copper Titration

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Much of the work dealing with porphyrins requires the handling of small amounts of pigments, usually of the order of micrograms. It is thus rarely possible to isolate a sufficient quantity of material of proved homogeneity suitable for carbon, hydrogen, oxygen or nitrogen analysis. Nevertheless, the highly characteristic absorption spectra of porphyrin pigments have enabled workers to make great progress in the field, as structural modifications are reflected strongly in the nature of the spectra.

In the present paper a problem of current interest concerning the porphyrin *a* isolated from the prosthetic group of cytochrome *a* of ox-heart muscle (Rawlinson & Hale, 1949) is taken as an illustration of the use of spectrophotometry in making accurate estimates of pigment concentrations.

The immediate aim was to determine molecular concentrations of porphyrin *a* for the calculation of molar extinction coefficients. The method described here utilizes the ability of free porphyrins to form metalloporphyrins which possess quite different light-absorbing properties. The conditions found most suitable involved the titration of free porphyrin in glacial acetic acid with a standard solution of cupric acetate in the same solvent. The formation of copper complex is quantitative, and no degradation of pigment is evident during the short period of heating necessary for the reaction. Although the disappearance of the red fluorescence of the free porphyrin, under ultraviolet radiation, can be used to give an approximate measure of the completion of the reaction, the end point is determined with much greater accuracy by measurements of the change of optical density at any wavelength where the two components have marked absorption differences. The reaction involves the replacement of the two hydrogen atoms bound to the nitrogen of each free porphyrin molecule by one cupric ion, so that the least number of metal ions required to produce the maximum change of optical density is equivalent to the number of free porphyrin molecules originally present.

The principles outlined might prove of use for the estimation of other pigments of unknown constitution, where quantitative conversion to another derivative of sufficiently different absorption spectrum is possible.

THEORY OF THE METHOD

If a series of dilutions be prepared containing constant amounts of porphyrin and gradually increasing concentrations of copper acetate, then, after completion of the reaction, the components present will be (i) free porphyrin, (ii) free porphyrin plus copper-porphyrin complex, or (iii) copper-porphyrin plus copper acetate.

The optical densities (D_1, D_2 and D_3) corresponding to these possibilities, at any selected wavelength, will be given by the equations,

$$D_1 = \{\epsilon_p c + b\}\, l, \quad (1)$$

$$D_2 = \{\epsilon_p (c - x) + \epsilon_m x + b\}\, l, \quad (2)$$

$$D_3 = \{\epsilon_m c + \epsilon_o (x - c) + b\}\, l, \quad (3)$$

where c is the initial molar concentration of porphyrin; x is the standard copper concentration in conditions (ii) and (iii); ϵ_p, ϵ_m and ϵ_o the molar extinction coefficients of free porphyrin, metallo-porphyrin, and copper acetate, respectively; b is any extraneous absorption of light, assumed constant for each wavelength; l is the thickness in cm. of solution absorbing light.

The combination of equations (1) and (2) gives an expression of condition (ii),

$$(D_2 - D_1) = x(\epsilon_m - \epsilon_p)\, l, \quad (4)$$

so that a plot of $(D_2 - D_1)$ against x will give a straight line passing through the origin with slope depending on $(\epsilon_m - \epsilon_p)$. It is important to select a wavelength giving greatest slope, and this may be done by preliminary inspection of absorption curves of the porphyrin and metal complex at equimolar concentrations (Fig. 1).

Beyond the end point the condition (iii) is expressed by combination of equations (1) and (3).

$$(D_3 - D_1) = \{c(\epsilon_m - \epsilon_p) + \epsilon_o (x - c)\}\, l, \quad (5)$$

from which it is obvious that any change in optical density will depend on x values, as all other terms are constant. Thus if $(D_3 - D_1)$ values are plotted against x, where it is certain that copper is present in excess, a straight line should result with slope dependent upon the small value, $\epsilon_o(x - c)$.

The end point is given by the point of intersection of the two straight lines defined by equations (4) and (5).

EXPERIMENTAL

Materials

Glacial acetic acid. British Drug Houses Ltd. analytical reagent, was recrystallized and distilled to eliminate metallic impurities.

Standard copper acetate solution (4×10^{-1} mM) was prepared by dissolving CuO (British Drug Houses Ltd. analytical reagent) in warm glacial acetic acid. For titration this solution was diluted to give a range of concentrations suitable for the selected amount of porphyrin.

Coproporphyrin III tetramethyl ester was isolated from cultures of *Corynebacterium diphtheriae*, and purified by the method of Gray & Holt (1947). The results presented in Fig. 3 were obtained with samples each containing $4{\cdot}22 \times 10^{-4}$ mmol. in glacial acetic acid.

Protoporphyrin IX was prepared from crystalline protohaemin by the method of Warburg & Negelein (1932) and recrystallized twice by mixing hot saturated solutions of the pigment in pyridine (1 vol.) and glacial acetic acid (10 vol.). The crystals were dried *in vacuo* over H_2SO_4 and then KOH. For the results quoted in Fig. 4 a $4{\cdot}72 \times 10^{-2}$ mM solution was prepared by dissolving the porphyrin in 1 vol. pyridine and then diluting to 100 vol. with glacial acetic acid. Samples were used for titration containing $4{\cdot}72 \times 10^{-4}$ mmol.

Porphyrin a was isolated from ox-heart muscle and purified by a modification of the method of Rawlinson & Hale (1949). After further purification the pigment was dissolved in $CHCl_3$ and stored at 2°. For titration, suitable samples were evaporated on the water bath and taken into a known volume of glacial acetic acid.

Methods

The sequence of steps involved in the estimation of porphyrin *a* is presented as a typical example of a pigment with unknown extinction coefficient data.

An approximate estimate of concentration is advisable if the minimum number of tubes is to be used in the more accurate method. The selection of suitable wavelengths is also necessary so as to obtain the greatest accuracy.

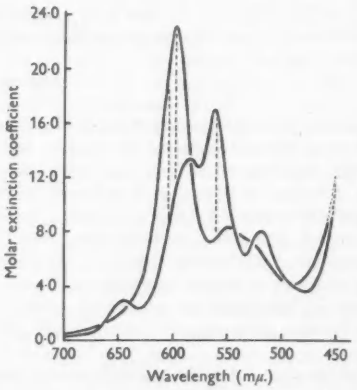

Fig. 1. Absorption spectra of porphyrin *a* and its copper complex. - - - - - -, Selected wave-lengths for titration.

(*a*) *Determination of approximate porphyrin concentration.* By evaporating a known volume of porphyrin *a* in chloroform and transferring into glacial acetic acid, the concentration of pigment was estimated roughly by direct titration with a standard copper acetate solution. The porphyrin solution was maintained at the temperature of the boiling-water bath and irradiated with ultraviolet light. The end point was taken as the disappearance of red fluor-

escence. At concentrations suitable for spectrophotometric work, the results of these fluorescence titrations tended to be too high, but at higher concentrations the method would probably be more accurate. This rough estimate of porphyrin concentration then allowed the preparation of suitable dilutions of standard copper acetate.

(*b*) *Selection of wavelengths suitable for measurements.* The next step required the selection of wavelengths giving large ($\epsilon_m - \epsilon_p$) values. After preliminary measurements to find a dilution of pigment where the optical density of the most intense absorption band was about 0·4, a sample was treated with a slight excess of standard copper acetate solution, and the original solution diluted to the same extent with glacial acetic acid. The absorption curves of the pigments were plotted, and then compared as shown in Fig. 1. The wavelengths most suitable for titration were readily selected (see broken lines at 604, 598 and 560 mμ.).

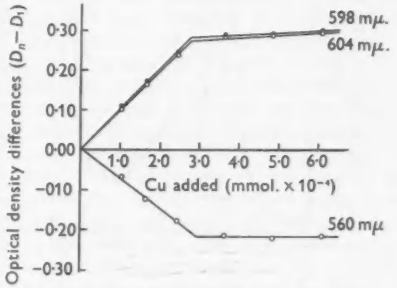

Fig. 2. Titration curves for porphyrin *a*.

(*c*) *Spectrophotometric method for accurate porphyrin estimations.* From the information derived above, 100 ml. of porphyrin *a* in glacial acetic acid was prepared so as to give suitable values of optical density, at the wavelengths selected. Ten ml. samples were pipetted into seven test tubes (6 × 1 in.) and to six of these were added 2 ml. portions of suitably diluted standard copper acetate solutions. The six standard dilutions of copper were selected to give a fairly uniform distribution of values, and arranged so that three points fell on each straight line (Fig. 2). To the seventh tube was added 2 ml. glacial acetic acid. The tubes were covered with glass funnels, heated on the boiling-water bath for 7 min., and then cooled. Each tube was then compared with glacial acetic acid in a Beckman spectrophotometer at 604, 598 and 560 mμ. and the results plotted as shown in Fig. 2. The end point was then measured as the point of intersection of the two 'best' straight lines calculated by the method of least squares.

Various tests on conditions of the method

(*a*) *Effect of heat on pigments.* Samples of porphyrin *a*, copper-porphyrin *a*, protoporphyrin, and copper-protoporphyrin were heated as described in the method. Samples were removed over a period of 15 min., cooled immediately and then measured in the spectrophotometer at wavelengths of maximum absorption. There was no significant change in optical density values, and so it must be concluded that the pigments were not modified during the heating process.

(*b*) *Quantitative estimation of coproporphyrin III ester.* This pigment was selected for the first test of the method as it was available in a pure state, and

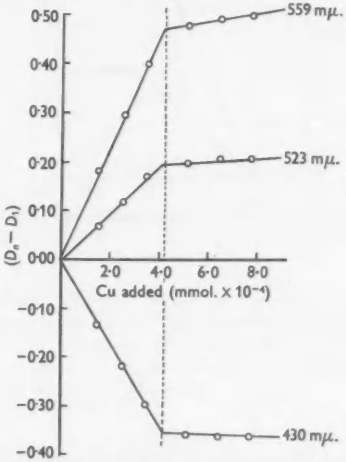

Fig. 3. Titration curves for coproporphyrin III tetra-methyl ester.

possessed stable peripheral groups. The result of the experiment is shown in Fig. 3, where the 'best' straight lines have been calculated. The point of intersection was obtained from the equations to the lines, and it was found to agree closely with the value expected (± 0·5 %).

(*c*) *Quantitative estimation of protoporphyrin IX.* The purpose in making this estimation was to test the method with a porphyrin possessing less stable substituents, in this case the vinyl groups at positions 2 and 4 being the cause of the increased lability. In addition, the porphyrin was used in the unesterified condition and so possessed free carboxyl groups. Another point of importance concerns the possibility of a close structural relationship between porphyrin *a* and protoporphyrin. Any difficulties associated with the estimation of the latter pigment would make it necessary to reserve judgement on the suitability of the method for porphyrin *a*. The results

illustrated in Fig. 4 show that the method gives a satisfactory estimate of concentration (± 2 %). The great difficulty in completing the removal of solvents from crystalline protoporphyrin was the main cause of error. Unless special care was taken in the removal of solvent, values as low as 80 % of theory were obtained. On account of the low solubility of protoporphyrin in glacial acetic acid, it was dissolved first in a small volume of pyridine before dilution. The final solutions of free porphyrin and

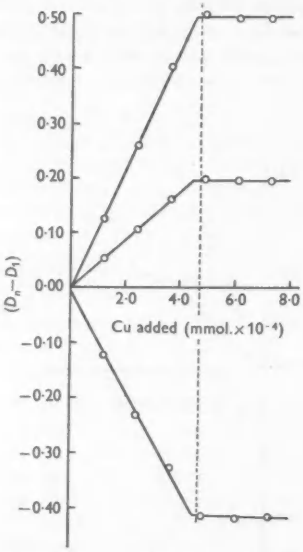

Fig. 4. Titration curves for protoporphyrin no. ix.

copper complex were tested for deviations from Beer's law, in case either pigment had commenced to polymerize. There was no evidence for this effect, as the optical density was found to be proportional to concentration in each case.

DISCUSSION

The spectrophotometric methods previously available for the estimation of porphyrins rely upon the data of extinction coefficients calculated from measurements on pure, crystalline compounds. As such information refers to a weight basis, the values are expressed as specific extinction coefficients. The method described in this paper allows of a direct estimate of molar concentration, without the necessity of making any assumptions as to either the molecular weight or extinction coefficients.

In the case of those porphyrins whose composition has not been established completely, values for carbon, hydrogen or oxygen cannot be used for estimation of molecular weight or molar concentration. The nitrogen content is a more feasible basis, as each porphyrin nucleus contains four nitrogen atoms, and no porphyrin isolated from natural sources has been found to possess additional nitrogen, with the exception of the prosthetic group of cytochrome *c*. Porphyrin *c* contains two cysteine residues (Theorell, 1939). However, even without accepting the assumption of four nitrogen atoms per molecule, the micro-analysis of nitrogen requires much greater amounts of pigment, is not so specific, and is not so convenient for general use. These comments indicate the main reasons for the development of the copper method. As reported earlier by Rawlinson & Hale (1949), the estimation of haemin *a* was possible on the basis of micro-iron analyses. The possibility of using the conversion of free porphyrins into metalloporphyrins has, therefore, been studied on a

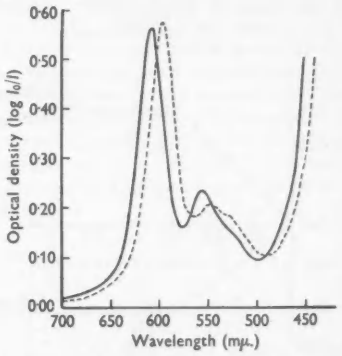

Fig. 5. Copper exchange in zinc porphyrin *a* complex. Zinc porphyrin *a*, ——; copper porphyrin *a*, - - -.

quantitative basis. Boas (1933) converted natural porphyrins into iron complexes and estimated these derivatives by their catalytic ability to oxidize benzidine.

Many heavy-metal ions can be made to replace the two hydrogens bound to the pyrrole nitrogen atoms of porphyrins. The speed of the reaction, and the stability of the metal complex depend on the nature of the metal ion, the porphyrin, and the solvent, as well as the temperature. Some idea of the firmness of the metal binding is given by reference to the competition between various ions and protons. Sodium, potassium and arsenic are removed by water. The magnesium and lead complexes are dissociated by dilute acetic acid. Concentrated hydrochloric acid causes the displacement of zinc, silver, tin (Sn^{++}) and iron (Fe^{++}). Concentrated sulphuric acid, on the other hand, is necessary to displace Fe^{+++}, Cu^{++}, Ni^{++}, Mn^{+++} and Co^{+++}, while tin (Sn^{++++}) and aluminium are not removed even under these conditions. It is also possible to displace one metal

by another; for example, the zinc-porphyrin *a* derivative can be converted into the copper complex when dissolved in a dilute solution of copper acetate in glacial acetic acid (Fig. 5).

The iron complex offers certain advantages as the iron or haemochrome contents could have served as a basis of estimation. However, there are some serious disadvantages. In particular, the ease of degradation to bile pigments, the ability to undergo reversible valency changes, and anhydride formation in hot organic solvents constitute the major reasons for avoiding the iron compounds. The direct analysis of heavy metal bound to porphyrin could be used, but would involve more work than the method here described.

There are several reasons which led to the choice of copper for complex formation. Standard solutions of copper acetate are readily prepared and the extremely stable copper-porphyrin forms under mild conditions. As the copper complexes do not fluoresce under ultraviolet radiation, the disappearance of red fluorescence is useful in making quick, preliminary estimates of concentration. In the new method where seven mixtures are compared, the plotting of the two straight lines is facilitated by grouping the results according as to whether or not there is red fluorescence. With some complexes, e.g. sodium, potassium, magnesium, zinc, cadmium and tin (Sn^{++}), this criterion is not available as both components fluoresce. The copper derivatives can be dissociated only by powerful reducing agents, such as sodium amalgam, and unlike the iron complexes do not appear to be capable of forming octahedral complexes by combination with other components of the reaction mixture. In glacial acetic acid the reaction between copper and porphyrin is irreversible.

The method was tested thoroughly by measurements on coproporphyrin ester and protoporphyrin, as well as by studies on the effect of heating and on the relationship between optical density and concentration for the least soluble components, namely, the protoporphyrin derivatives. The use of glacial acetic acid requires some care on account of its corrosive nature, but in spite of this fact it is by far the most satisfactory solvent. The reaction when carried out in pyridine requires longer heating with consequent dangers of loss of solvent and modification of pigments. In particular porphyrin *a*, with its labile formyl group, should be kept in an acidic environment.

Some emphasis could also be laid on the specificity of the method. The elimination of the term *b* in the final equations (4) and (5) means that any component affecting the transmission of incident light need not be considered if the effect remains constant during the conditions of reaction.

Any contaminating substance that forms an irreversible complex with copper would compete with porphyrin and so change the slope of the curve defined as $(\epsilon_m - \epsilon_p)$ until it was all bound to copper (Fig. 6). However, by extrapolating back the straight line beyond this deviation, the intercept on the copper axis would give a true indication of the end point.

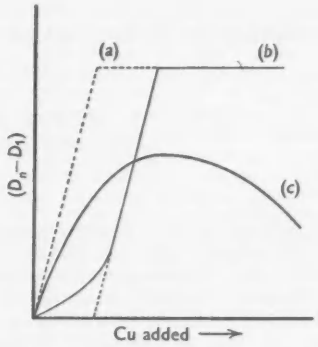

Fig. 6. Effect of some interfering factors on the shape of the titration curve: (*a*) theoretical curve; (*b*) copper-binding impurities; (*c*) copper-catalysed decomposition.

Another source of error that requires comment is the possibility of decomposition catalysed by copper. In such a case the titration curve would take the form shown in Fig. 6, but in none of our titrations has this effect been observed.

SUMMARY

1. For the micro-estimation of porphyrin *a*, a new spectrophotometric method, which should prove of more general application, has been developed.

2. The method involves the titration of porphyrin with standard copper solutions by following the changes of optical density at selected wavelengths where the free porphyrin and copper porphyrin complex show greatest differences of absorption.

3. The accuracy of the method has been demonstrated by reference to pure specimens of coproporphyrin III tetramethyl ester and protoporphyrin, under different experimental conditions, and the effects of interfering reactions have been considered.

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REFERENCES

Boas, I. (1933). *Dtsch. med. Wschr.* **59**, 126.
Gray, C. H. & Holt, L. B. (1947). *J. biol. Chem.* **169**, 235.
Rawlinson, W. A. & Hale, J. H. (1949). *Biochem. J.* **45**, 247.
Theorell, H. (1939). *Enzymologia*, **6**, 88.
Warburg, O. & Negelein, E. (1932). *Biochem. Z.* **244**, 9.

The Metabolic Reduction of Progesterone to *allo*Pregnane-3α:20α-diol in Human Subjects

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Since the original isolation of *allo*pregnane-3α:20α-diol from the urine of pregnant women by Hartmann & Locher (1935) it has been generally assumed that this steroid, like its C-5 stereoisomer pregnane-3α:20α-diol, is a metabolic reduction product of progesterone. Hitherto, however, no satisfactory experimental evidence to warrant this assumption has been published.*

The isolation of *allo*pregnane-3α:20α-diol from the urine of a post-menopausal woman receiving progesterone treatment has recently been reported from this laboratory (Kyle & Marrian, 1951), but since control urine collected from the same subject before or after the treatment was not examined, it could not be concluded that the isolated steroid had necessarily arisen by reduction of the administered hormone. The possibility that the *allo*pregnane-3α:20α-diol had originated from some endogenous source, and otherwise than by the reduction of progesterone, did not seem to be entirely remote in view of the reported findings of Dobriner, Lieberman, Rhoads & Taylor (1948). These workers, in a study of the ketosteroid excretion of a pregnant woman, showed that whereas the urinary pregnan-3α-ol-20-one increased steadily from the third month until term, the excretion of *allo*pregnan-3α-ol-20-one excretion remained relatively constant at about 1–2 mg./24 hr. throughout pregnancy. In view of these findings the possibility could not be entirely ignored that the urinary *allo*pregnane steroids, in contrast to those of the pregnane series, might arise from some precursor other than progesterone.

In the present work *allo*pregnane-3α:20α-diol has been isolated from the urine of one normal post-menopausal woman and one rheumatoid arthritic man who were being treated daily with large doses of progesterone by intramuscular injection. In both cases urine specimens were collected over adequate control periods after the cessation of the hormone treatment and were examined by methods as nearly identical as possible with those used for the 'progesterone' urines. In neither instance could any *allo*pregnane-3α:20α-diol be isolated from these control urines, and it can therefore be concluded that the material isolated from the 'progesterone' urines had indeed been formed by the reduction of the administered progesterone.

It is of some considerable interest that the relative proportions of pregnane-3α:20α-diol and *allo*pregnane-3α:20α-diol isolated from the urine during periods of progesterone administration in these experiments (approx. 30:1) agree very closely with the relative proportion of these two steroids isolated from the urine of pregnant women by Kyle & Marrian (1951). This suggests that the *allo*-pregnane-3α:20α-diol present in the urine during pregnancy arises largely by the metabolic reduction of endogenous progesterone.

EXPERIMENTAL

Methods

Melting points were determined as previously described (Kyle & Marrian, 1951). Al_2O_3 (Peter Spence and Sons) used for chromatography was activated to activity II, as determined by the method of Brockmann & Schodder (1941), by heating to 100° *in vacuo*. In most cases the amount of Al_2O_3 used was 30 times the weight of the fraction to be chromatographed.

All solvents were dried and redistilled before use.

Collection, extraction and fractionation of urine specimens

Urine was collected daily from each case using toluene as preservative, and hydrolysed by boiling under reflux for

* Since the completion of the present work Dorfman & Unger (1950) have reported that they have demonstrated the conversion of progesterone into *allo*pregnane-3α:20α-diol in human subjects. Experimental details of this work are not yet available, however.

10 min. after the addition of 10 vol. % of conc. HCl. The 'neutral ether-soluble fraction' was prepared daily from each hydrolysed specimen in the usual way by extraction with ether, washing the extract with aqueous NaOH and with water, and evaporation of the washed extract to dryness. The neutral ether-soluble fractions obtained from each case were bulked at the end of each experimental period ('progesterone' or control), before further working up.

Each bulked neutral ether-soluble fraction was dissolved in benzene and poured through a column of Al_2O_3. After exhaustive washing with benzene and with ether, the column was eluted with acetone until evaporation of portions of the eluate yielded little or no solid or gummy material. Evaporation of the combined acetone eluates yielded the 'crude diol fraction'.

First experiment

The first experiment was carried out upon a normal post-menopausal woman who was treated with 50 mg. progesterone/day for 22 days by intramuscular injection.

'*Progesterone*' *period.* Complete 24 hr. specimens of urine were collected throughout the period of progesterone treatment. Two-fifths of each specimen were used for another purpose, so that the urine available for the present study was equivalent to 13·2 twenty-four hr. specimens only.

The crude diol fraction (180 mg.) which was a dark-purple semi-crystalline solid, was refluxed for 1 hr. with 25 ml. benzene, and after standing overnight at room temperature the solid material was filtered off. Evaporation of the benzene filtrate yielded 117 mg. of a dark-red gum which was dissolved in 2 ml. methanol. After standing for several days at room temperature the solution deposited crystalline material. The latter was filtered off and recrystallized from about 1·5 ml. methanol when 1·9 mg. of thick crystalline rods melting at 240–244° were obtained. The melting point was unchanged on admixture with authentic *allo*pregnane-3α:20α-diol (Kyle & Marrian, 1951), but was depressed 20° on admixture with pregnane-3α:20α-diol. The identity of the isolated material was confirmed by the preparation of an acetate, the melting point of which (136–140°) was not depressed on admixture with authentic *allo*pregnane-3α:20α-diol diacetate (m.p. 137–139°).

The white solid material filtered off after the treatment of the crude diol fraction with benzene (63 mg.), was treated with benzene a second time in the same manner as before. The residue obtained on evaporation of the second benzene filtrate yielded a small amount of pregnane-3α:20α-diol but no *allo*pregnane-3α:20α-diol. The main bulk of the benzene-insoluble material was unfortunately not examined further, but since the corresponding fraction obtained in the 'progesterone' period of the second experiment (see below) proved to be nearly pure pregnane-3α:20α-diol, there is good reason to believe that this material also consisted largely of pregnane-3α:20α-diol. The ratio of the amounts of pregnane-3α:20α-diol and *allo*pregnane-3α:20α-diol isolated in this experiment was therefore 63:1·9 = 33:1.

Control period. Commencing 1 week after the last dose of progesterone seventeen successive complete 24 hr. urine specimens were collected from the same patient.

The crude diol fraction (105 mg.) was a dark-purple oil. On refluxing with 25 ml. benzene a clear solution was obtained from which no solid was deposited after standing overnight at room temperature.

Because of the oily character of the diol fraction it was decided to refractionate it chromatographically before examination for the presence of *allo*pregnane-3α:20α-diol. Accordingly, the benzene solution was poured on to a column of Al_2O_3 which was then eluted successively with benzene (100 ml.), 25% (v/v) ether in benzene (30 ml.), 50% (v/v) ether in benzene (45 ml.), ether (75 ml.), 2% (v/v) acetone in ether (45 ml.), 5% (v/v) acetone in ether (45 ml.), 50% (v/v) acetone in ether (75 ml.), and acetone (60 ml.). Successive 15 ml. volumes of the eluates were evaporated to dryness and the residues examined for the presence of crystalline material by adding 1 or 2 drops of acetone and allowing to stand overnight.

Crystalline material was detected only in the first 15 ml. fraction eluted by 50% acetone in ether. After leaching this fraction with ice-cold acetone, a very small amount of white crystalline material remained which melted at 231–234°. The mixed melting point with authentic pregnane-3α:20α-diol was 231–235°, while that with authentic *allo*pregnane-3α:20α-diol was 215–228°. High vacuum sublimation of the acetone leachings of this fraction yielded an additional small amount of crystalline material melting at 232–238°. The mixed melting point of this with authentic pregnane-3α:20α-diol was 230–235°, while that with authentic *allo*-pregnane-3α:20α-diol was 215–230°.

The isolation of a small amount of pregnane-3α:20α-diol from the control urine of this patient so long after the cessation of the progesterone treatment occasioned no surprise, since this steroid has been isolated in minute amounts from the urine of untreated men (Westphal, 1944) and from the urine of a post-menopausal women who had received no previous treatment with progesterone (Marrian, 1948).

No *allo*pregnane-3α:20α-diol could be detected in any of the fractions obtained from the chromatogram.

Second experiment

The subject for this experiment was a rheumatoid arthritic man who was treated with 100 mg. progesterone/day for 14 days by intramuscular injection.

'*Progesterone period*'. Complete 24 hr. urine specimens were collected throughout the period of progesterone treatment. The crude diol fraction obtained (680 mg.) was refluxed for 1 hr. with 25 ml. benzene, and after standing overnight at room temperature the solid was filtered off. The benzene filtrate was poured onto a column of Al_2O_3 which was then developed with benzene (300 ml.) and ether (100 ml.). The washed column was then eluted with 5% (v/v) acetone in ether (50 ml.), 50% (v/v) acetone in ether (100 ml.), and acetone (150 ml.). Successive 25 ml. portions of the eluates were evaporated to dryness. Crystalline material was detected by 'spotting' with acetone in the last five fractions eluted by ether-acetone and in the first eluted by acetone. These were combined, dissolved in the minimum amount of hot methanol, and the solution set aside in the refrigerator. After several days the thick crystalline rods which had separated were filtered off. These weighed 4·5 mg. and melted at 243–244°. On admixture with authentic *allo*-pregnane-3α:20α-diol the melting point was unchanged, while on admixture with authentic pregnane-3α:20α diol it

was depressed to 215–218°. No further *allo*pregnane 3α:20α diol could be obtained from the methanol mother liquors or from a second benzene leaching of the solid material in the diol fraction which had not dissolved in the first benzene treatment.

This benzene-insoluble material (120 mg.), although slightly coloured, appeared to consist largely of pregnane-3α:20α-diol, since it melted at 234–235° and since the mixed melting point with authentic pregnane-3α:20α-diol was 234–236°.

The ratio of pregnane-3α:20α-diol to *allo*pregnane-3α:20α-diol isolated in this experiment was therefore 120:4·5 = 27:1.

First control period. Commencing 2 days after the cessation of the progesterone treatment fourteen complete 24 hr. urine samples were collected. The crude diol fraction (215 mg.), which was a red gum, was refluxed with 25 ml. benzene; it was completely soluble and no solid separated from the solution on standing overnight. This benzene solution was then chromatographed in an identical manner to that described above. Crystalline material was detected only in the second and third fractions eluted with 50% (v/v) ether-acetone. These were combined and crystallized from the minimum volume of methanol, when a few mg. of fine needles with m.p. 234–235° were obtained. A further small amount of crystalline material of m.p. 234–235° was obtained from the methanol mother liquor by high vacuum sublimation and crystallization of the sublimate from acetone. These melting points were not depressed on admixture with authentic pregnane-3α:20α-diol. No indication of the presence of even traces of *allo*pregnane-3α:20α-diol could be detected in any of the chromatogram fractions.

Second control period. The failure to detect *allo*pregnane-3α:20α-diol in the control period urines in both experiments was not considered to provide entirely conclusive evidence that the steroid was indeed not present in amounts comparable to those found in the 'progesterone' period urines: the possibility had to be considered that in the presence of large amounts of pregnane-3α:20α-diol the solubilities and elutability of *allo*pregnane-3α:20α-diol might be so altered as to facilitate greatly its isolation. Accordingly, in this experiment it was decided to work up a second batch of control urine adding pure pregnane-3α:20α-diol to the neutral ether-soluble fraction before separating the diol fraction by chromatography.

Two months after the cessation of the progesterone treatment a further fourteen consecutive 24 hr. urine samples were collected from the same patient. To the neutral ether-soluble fraction 300 mg. of carefully purified pregnane-3α:20α-diol was added.

The crude diol fraction was a light-brown crystalline solid weighing 790 mg. It was refluxed with 25 ml. benzene for 2 hr. and after standing overnight at room temperature the solid material was filtered off. The benzene filtrate was poured on to a column of Al_2O_3 which was then developed with benzene (200 ml.) and ether (200 ml.). The washed column was then eluted with 5% (v/v) acetone in ether (50 ml.), 8% (v/v) acetone in ether (50 ml.), 10% (v/v) acetone in ether (25 ml.), 50% (v/v) acetone in ether (100 ml.) and acetone (75 ml.). Successive 25 ml. portions of the eluates were evaporated to dryness and 'spotted' with acetone.

Crystalline material was detected only in the first and second portions of the 50% acetone in ether eluate. These were combined, dissolved in the minimum volume of methanol and the solution left in the refrigerator for 1 week to crystallize. The crystals which separated melted at 230–234° and on admixture with authentic pregnane-3α:20α-diol the melting point was 233–235°. It was clear, therefore, that in the control urine *allo*pregnane-3α:20α-diol was not present in an amount comparable to that found in the urine of the same patient during treatment with progesterone.

SUMMARY

1. *allo*Pregnane-3α:20α-diol has been isolated from the urines of one post-menopausal woman and one rheumatoid arthritic man collected during treatment with progesterone. The weights of *allo*pregnane-3α:20α-diol isolated corresponded in each case to about 0·3% of the amount of progesterone administered.

2. No trace of *allo*pregnane-3α:20α-diol could be detected in control urines collected from the same patients, and accordingly it is concluded that the *allo*pregnane-3α:20α-diol was formed by the metabolic reduction of the administered progesterone.

3. The proportions of pregnane-3α:20α-diol and *allo*pregnane-3α:20α-diol isolated after progesterone administration are similar to those found in the urine of pregnant women (Kyle & Marrian, 1951).

REFERENCES

Brockmann, H. & Schodder, F. (1941). *Ber. dtsch. chem. Ges.* **74**, 73.

Dobriner, K., Lieberman, S., Rhoads, C. P. & Taylor, H. C. (1948). *Obstet. Gynec. Survey*, **3**, 677.

Dorfman, R. I. & Unger, F. (1950). *XVIIIth Int. Physiol. Congr. Abstr.* p. 178.

Hartman, M. & Locher, F. (1935). *Helv. chim. Acta*, **18**, 160.

Kyle, T. I. & Marrian, G. F. (1951). *Biochem. J.* (in the Press).

Marrian, G. F. (1948). Unpublished observation.

Westphal, U. (1944). *Hoppe-Seyl. Z.* **281**, 14.

Biochemical Studies of Toxic Agents

3. THE ISOLATION OF 1- AND 2-NAPHTHYLSULPHURIC ACID AND 1- AND 2-NAPHTHYLGLUCURONIDE FROM THE URINE OF RATS DOSED WITH 1- AND 2-NAPHTHOL

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The determination of the so-called 'ethereal sulphate content' of urine is a simple analytical procedure, and there are many reports in the literature which show that the administration of phenols to experimental animals is followed by an increased excretion of ethereal sulphates in the urine. On the other hand, because of the difficulties which have usually been encountered, relatively few ethereal sulphates have been separated from urine. For many phenols, therefore, the belief that they are converted to arylsulphuric acids in the body is based solely on the observation that when administered to animals they give rise to an increase in the ethereal sulphate content of the urine.

In the free state many arylsulphuric acids are unstable substances, and those which have been isolated from urine have usually been separated in the form of their alkali-metal salts. The separation of such salts from urine is not easily accomplished, however. In the course of investigations designed to find an alternative method of isolating arylsulphuric acids, Laughland & Young (1942) obtained evidence that these compounds can be separated from urine in the form of their salts with organic bases. At the same time, Barton & Young (1943) developed a simple method of preparing salts of this type, and in a study of the salts formed by a series of arylsulphuric acids with various organic bases they found that *p*-toluidine consistently yielded crystalline derivatives with properties well suited to the characterization of the arylsulphuric acids they were studying. Later work by Laughland & Young (1944) showed that the *p*-bromoaniline salts of a number of arylsulphuric acids have a low solubility in water. One outcome of these investigations was that other workers were led to employ organic bases in the separation and characterization of urinary steroid sulphates (cf. Klyne & Marrian, 1945; Klyne, Schachter & Marrian, 1948; Paterson & Klyne, 1948).

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It has long been known that when 1- and 2-naphthol are administered to dogs these compounds are excreted, in part, as glucuronides (Lesnik & Nencki, 1886). Furthermore, the increased excretion of ethereal sulphates which occurs under these conditions has led to the belief that 1- and 2-naphthol are also excreted in conjugation with sulphuric acid (Mauthner, 1881; Lesnik & Nencki, 1886). The isolation of 1- and 2-naphthylsulphuric acid from urine has not been described, however, and the present work was undertaken in order to determine whether these substances could be separated from the urine of animals dosed with 1- and 2-naphthol. This has now been achieved by the isolation of 1-naphthylsulphuric acid (as its *p*-bromoaniline salt) from the urine of rats dosed with 1-naphthol, and by the isolation of 2-naphthylsulphuric acid (as its *p*-toluidine salt) from the urine of rats dosed with 2-naphthol. In the course of isolating the naphthylsulphuric acids it was also found that 1- and 2-naphthylglucuronide can be separated in the form of their *p*-toluidine salts from the urine of rats dosed with 1- and 2-naphthol, respectively. The free naphthols were also isolated from the urine of the dosed animals.

THE METABOLISM OF 1-NAPHTHOL

General methods. Six male and six female white rats weighing between 180 and 220 g. were used in each experiment. The animals were housed in metabolism cages which permitted the collection of urine separate from the faeces. They were fed once a day for a period of 2 hr. on a diet which consisted of Master Fox Breeding Ration (Toronto Elevators Ltd.), supplemented with fresh milk and brown bread. The urine excreted during the feeding period was not collected. Water was available to the animals at all times.

The 1-naphthol was purified by distillation at reduced pressure and was administered to the rats in the form of a 20% (w/v) solution in corn oil. Solutions of 1-naphthol in corn oil darken on standing and a fresh solution was prepared on each day that the animals were dosed. Each rat was given 0·67 ml. of this solution by injection under the skin of the back immediately after the feeding period on 4 successive days. The collection of urine was begun immediately after the first injection and was continued until 2 days had elapsed

after the last injection. The urine was collected each day and was stored in the refrigerator until the end of the experimental period, and it was then used for the isolation procedures described below. The total amount of 1-naphthol administered in each experiment was 6·4 g.

Isolation of 1-naphthol from urine

The urine (pH 6–7) was extracted with peroxide-free ether in a continuous extractor for a total period of 24 hr. The ether extract was evaporated to dryness on a water bath and the residue was sublimed at 65–75° under reduced pressure. The sublimate was crystallized from *cyclo*hexane and yielded an almost colourless crystalline product; m.p. 94–95°; mixed m.p. with 1-naphthol, 94–95° (these and other melting points reported herein are uncorrected). (Found: C, 83·0; H, 5·7. Calc. for $C_{10}H_8O$: C, 83·3; H, 5·6%.)

In three experiments the weights of 1-naphthol isolated from the urine were 0·163, 0·152 and 0·187 g. These amounts represented 2·5, 2·4 and 2·8%, respectively, of the 1-naphthol injected.

Isolation of 1-naphthylglucuronide from urine

The urine, after the removal of 1-naphthol by the procedure already described, was adjusted to pH 8–9 by the addition of 20% (w/v) NaOH solution. It was then evaporated at 50–60° under reduced pressure to one-fifth of its original volume of 400–500 ml. The concentrated urine was then transferred to a 250 ml. centrifuge bottle and extracted with seven 100 ml. portions of *n*-butanol. The urine was shaken with each portion of butanol for 15 min. on a mechanical shaker, the mixture was then centrifuged, the butanol layer was withdrawn, and 10 ml. of water were added to the urine to maintain its volume. The butanol extracts were combined and evaporated to dryness at 50–60° under reduced pressure. The residue was dissolved in 50 ml. of water at 60–70° and to the solution at this temperature 2·0 g. of solid *p*-toluidine hydrochloride were added with stirring. The solution was allowed to cool to room temperature and yielded a bulky crystalline precipitate. The mixture was left in the refrigerator overnight, filtered, and the precipitate was dissolved in hot water. Charcoal was added to the solution, which was then heated and filtered. The filtrate was allowed to cool to room temperature and was then left overnight in the refrigerator. The white crystalline product was filtered and dried over P_2O_5 *in vacuo* at room temperature.

Properties of the compound. The product was almost insoluble in cold water, but was quite soluble in hot water. It melted with decomposition at 154–156° with previous darkening. This melting point was not changed when the material was recrystallized several times from water. In order to determine the melting point of the compound an approximate melting point was first obtained in the usual way, and the melting point was then determined with a sample introduced into the bath at a temperature 10° below the approximate melting point. (Found: C, 64·4; H, 6·1; N, 3·1. $C_{23}H_{25}O_7N$ requires C, 64·7; H, 5·9; N, 3·3%.) Neutralization equivalent found, 430; calc. for *p-toluidine 1-naphthylglucuronidate*, 427. The compound gave a positive naphthoresorcinol test for glucuronide. It gave a positive test with Benedict's reagent after, but not before, it had been boiled with concentrated hydrochloric acid.

Decomposition of the compound with alkali. 10% (w/v) NaOH (10 ml.) was added to 1·0 g. of the compound. The mixture was stirred well and was extracted with three 30 ml. portions of ether. The ether extracts were combined, dried over anhydrous $CaCl_2$ and filtered. The filtrate was evaporated to dryness and the residue was dissolved in hot 95% ethanol. The solution was heated with charcoal and filtered, and the filtrate was diluted with water. The mixture was allowed to stand in the refrigerator and the precipitate was filtered. The product weighed 0·185 g.; m.p. 45–46°; mixed m.p. with *p*-toluidine, 45–46°.

Decomposition of the compound with acid. 6N-HCl (10 ml.) was added to 1·0 g. of the compound and the mixture was stirred. The compound dissolved and in a short time a precipitate began to settle out. The mixture was left in the refrigerator for several hours and on filtration it yielded 0·730 g. of crystalline product. This was recrystallized from water; m.p. 198–199°; $[\alpha]_D^{20°} - 85°$ in ethanol (*c*, 1). (Found: C, 55·2; H, 5·6. Calc. for $C_{16}H_{16}O_7 . 1·5\ H_2O$: C, 55·3; H, 5·5%.) Neutralization equivalent found, 350; calc. for 1-naphthylglucuronide, 347. Lesnik & Nencki (1886) reported that the 1-naphthylglucuronide, which they isolated from the urine of animals dosed with 1-naphthol, melted at 202–203°.

The compound (1 g.) obtained by decomposition of the *p*-toluidine salt with acid was heated under reflux on a boiling-water bath with 20 ml. of N-H_2SO_4 for 6 hr. The hydrolysate was cooled, left overnight in the refrigerator, and the precipitate was separated by filtration. The filtrate was extracted with three 30 ml. portions of ether, the combined ether extracts were dried over anhydrous $CaCl_2$, filtered and evaporated to dryness. The residue, together with the precipitate from the hydrolysate, was sublimed at about 80° under reduced pressure. This yielded 0·265 g. of sublimate, m.p. 93–94°. When this material was mixed with 1-naphthol, the melting point was not depressed.

Amounts of compound isolated. The amounts of *p*-toluidine 1-naphthylglucuronidate isolated from the urine were 2·8, 2·9 and 3·2 g., and these corresponded to 14·7, 15·2 and 16·8%, respectively, of the 1-naphthol administered.

Isolation of 1-naphthylsulphuric acid from urine

The urine collected from twelve rats which had received a total of 6·4 g. of 1-naphthol by subcutaneous injection was extracted with ether and with *n*-butanol by the methods already described. The combined butanol extracts were evaporated to dryness at 50–60° under reduced pressure. The residue obtained on evaporation of the butanol was dissolved in 60 ml. of water at 60–70° and 2·0 g. of *p*-bromoaniline hydrochloride were added with stirring. The mixture was left overnight in the refrigerator and was then filtered. The precipitate, *P*-1, consisted chiefly of *p*-bromoaniline 1-naphthylglucuronidate and it gave a slightly positive test for ethereal sulphate. The filtrate, *F*-1, gave a strongly positive test for ethereal sulphate. *P*-1 was dissolved in hot water, and the hot solution was treated with charcoal and filtered. The filtrate obtained was left in the refrigerator and

was then filtered. The precipitate, *P*-2, gave no test for ethereal sulphate, whereas the filtrate, *F*-2, gave tests for the presence of ethereal sulphate and glucuronidate. *F*-1 and *F*-2 were combined and evaporated to 40 ml. under reduced pressure. The concentrate was left in the refrigerator and when filtered it yielded a precipitate, *P*-3, which consisted of glucuronidate. The filtrate, *F*-3, obtained after separation of *P*-3, was evaporated to dryness at 50–60° under reduced pressure. The residue obtained after evaporation of *F*-3 was extracted with two 20 ml. portions of boiling ethanol, and the ethanol extracts were cooled and filtered. The precipitate, *P*-4, contained no ethereal sulphate. The filtrate, *F*-4, was evaporated to dryness at 50–60° under reduced pressure. The residue was dissolved in the minimum amount of hot water, and the solution was heated with charcoal and filtered through a heated funnel. The filtrate was cooled in the refrigerator and filtered. It yielded a precipitate which consisted of *p*-bromoaniline 1-naphthylsulphate contaminated with *p*-bromoaniline 1-naphthylglucuronidate. The precipitate was recrystallized three times from water and a product free from glucuronidate was obtained.

Properties of the compound. The compound gave positive tests for sulphate and for naphthol after it had been hydrolysed with hydrochloric acid, but not before it had been hydrolysed. (Found: C, 48·5; H, 3·7; N, 3·3. Calc. for $C_{16}H_{14}O_4NSBr$: C, 48·5; H, 3·5; N, 3·5%.) Neutralization equivalent found, 393; calc. for *p*-bromoaniline 1-naphthylsulphate, 396.

Potassium 1-naphthylsulphate was synthesized by the method of Feigenbaum & Neuberg (1941), and 1·0 g. of this compound was converted to the *p*-bromoaniline salt by a procedure similar to that described by Barton & Young (1943). This yielded 0·820 g. of purified product. (Found: C, 48·6; H, 3·6; N, 3·4. Calc. for $C_{16}H_{14}O_4NSBr$: C, 48·5; H, 3·5; N, 3·5%.) The melting point of this compound was ill-defined, as also was the melting point of the *p*-bromoaniline salt of the ethereal sulphate isolated from urine (cf. Laughland & Young, 1944).

Hydrolysis of the compound. 6N-HCl (5 ml.) was added to 0·100 g. of the compound obtained from urine and the mixture was heated in a boiling-water bath for 30 min. The hydrolysate was allowed to cool to room temperature and was then extracted with three 30 ml. portions of ether. The ether extracts were combined, washed with water and dried over anhydrous $CaCl_2$. The ether solution was filtered, the filtrate was evaporated to dryness and the residue was sublimed under reduced pressure. The sublimate, 0·024 g., melted at 94–95°, and the melting point was not depressed when the compound was mixed with 1-naphthol.

Amounts of the compound isolated. In three experiments the amounts of *p*-bromoaniline 1-naphthylsulphate isolated from the urine of the dosed animals were 0·063, 0·087 and 0·098 g., and these corresponded to 0·4, 0·5 and 0·6%, respectively, of the 1-naphthol administered.

THE METABOLISM OF 2-NAPHTHOL

General methods. These were similar to those employed in studying the metabolism of 1-naphthol, except that 2-naphthol was administered as a 12·5% (w/v) solution in corn oil. Each rat received 1·0 ml. of this solution by subcutaneous injection on each of the first 2 days of the experiment and 0·5 ml. of the solution on each of the next 2 days. The total amount of 2-naphthol administered to the group of twelve rats used in each experiment was 4·5 g.

Isolation of 2-naphthol from urine

The urine (pH 6–7) was extracted in a continuous extractor with peroxide-free ether for a total period of 24 hr. The residue obtained on evaporation of the ether extract was dissolved in hot water and the solution was heated with charcoal. The charcoal was removed by filtration and the filtrate was left overnight in the refrigerator. It yielded a colourless crystalline precipitate, m.p. 121–122°, mixed m.p. with 2-naphthol 121–122°. (Found: C, 83·2; H, 5·7. Calc. for $C_{10}H_8O$: C, 83·3; H, 5·6%.)

In two experiments the amounts of 2-naphthol isolated from the urine were 0·404 and 0·610 g. These amounts represented 9·0 and 13·5%, respectively, of the 2-naphthol administered.

Isolation of 2-naphthylglucuronide from urine

2-Naphthylglucuronide was separated from urine in the form of its *p*-toluidine salt by the same procedure as was used for the isolation of 1-naphthylglucuronide.

Properties of the compound. The *p*-toluidine salts of 1- and 2-naphthylglucuronide showed similar properties. The p-*toluidine* 2-*naphthylglucuronidate* melted at 184–186°. (Found: C, 64·8; H, 5·7; N, 3·2; neutralization equivalent, 429. $C_{23}H_{25}O_7N$ requires C, 64·7; H, 5·9; N, 3·3%; neutralization equivalent, 427.) The compound gave a positive naphthoresorcinol test for glucuronide. It reduced Benedict's reagent after, but not before, it had been hydrolysed with concentrated hydrochloric acid.

Decomposition of the compound with alkali. When 1·0 g. of the compound was decomposed with alkali by the procedure already described for the alkaline decomposition of *p*-toluidine 1-naphthylglucuronidate, 0·182 g. of *p*-toluidine, m.p. and mixed m.p. 45–46°, was obtained.

Decomposition of the compound with acid. When 10 ml. of 6N-HCl were added, with stirring, to 1·0 g. of the compound, a solution was obtained which on standing yielded a precipitate. The mixture was left in the refrigerator for several hours and the precipitate was then separated by filtration. The precipitate, after prolonged drying over P_2O_5 *in vacuo*, weighed 0·610 g., m.p. 149–150°, $[\alpha]_D^{22°}$ – 97° in ethanol (*c*, 1%). (Found: C, 59·9; H, 5·3. Calc. for $C_{16}H_{16}O_7$: C, 60·0; H, 5·0%.) The anhydrous 2-naphthylglucuronide obtained by Lesnik & Nencki (1886), from the urine of dogs dosed with 2-naphthol, melted at 150°.

The compound (1 g.) prepared by the acid decomposition of the *p*-toluidine salt by the method just described was refluxed with 20 ml. of N-H_2SO_4 for 6 hr. The hydrolysate was left in the refrigerator overnight and the precipitate which formed was removed by filtration. The filtrate was extracted with three 30 ml. portions of ether and the combined ether extracts were dried over anhydrous $CaCl_2$ and filtered. The ether solution was evaporated and the residue, together with the precipitate obtained after hydrolysis, was sublimed under reduced pressure. The sublimate weighed 0·312 g.; m.p. 120–121°; mixed m.p. with 2-naphthol, 120–121°.

Amounts of the compound isolated. The amounts of the *p*-toluidine salt of 2-naphthylglucuronide isolated in three experiments were 2·4, 2·5 and 3·0 g., and these corresponded to 18·0, 18·7 and 22·5%, respectively, of the 2-naphthol administered.

Isolation of 2-naphthylsulphuric acid from urine

The procedure used for the isolation of 2-naphthylsulphuric acid from urine resembled closely that already described for the isolation of 1-naphthylsulphuric acid, except that the compound was isolated in the form of the *p*-toluidine salt instead of the *p*-bromoaniline salt.

Properties of the compound. The compound obtained from urine gave positive tests for sulphate and for naphthol after, but not before, it had been hydrolysed with hydrochloric acid. It melted at 156–158° with decomposition, and the mixed melting point with synthetic p-*toluidine 2-naphthylsulphate* was not depressed. (Found: C, 61·7; H, 5·3; N, 4·0. $C_{17}H_{17}O_4NS$ requires C, 61·6; H, 5·1; N, 4·2%.) Neutralization equivalent found, 327; calc. for *p*-toluidine 2-naphthylsulphate, 331.

Potassium 2-naphthylsulphate was synthesized by the method of Feigenbaum & Neuberg (1941), and 1·0 g. of this compound was converted to the *p*-toluidine salt (0·745 g.) by the method of Barton & Young (1943). The product melted with decomposition at 156–158°. (Found: C, 61·8; H, 5·3; N, 4·3. Calc. for $C_{17}H_{17}O_4NS$: C, 61·6; H, 5·1; N, 4·2%.)

Hydrolysis of the compound. The compound (0·100 g.) obtained from urine was heated in a boiling-water bath with 5 ml. of 6N-HCl for 30 min. The solution was left in the refrigerator for 3 hr., and the precipitate which formed was filtered and crystallized from water. The product, 0·028 g., melted at 121–122°, and the mixed melting point with 2-naphthol was not depressed.

Amounts of the compound isolated. The amounts of *p*-toluidine 2-naphthylsulphate isolated from the urine in three experiments were 0·165, 0·187 and 0·204 g. These quantities were derived from 1·6, 1·8 and 2·0%, respectively, of the 2-naphthol injected.

DISCUSSION

Although it has long been accepted that excretion of 1- and 2-naphthylsulphuric acid takes place following the administration of 1- and 2-naphthol, respectively, the isolation of the conjugated compounds from urine does not appear to have been described. The isolation of 1- and 2-naphthylsulphuric acid from the urine of rats dosed with the corresponding naphthols has now been carried out by making use of the fact that salts of arylsulphuric acids react in aqueous solution with the hydrochlorides of organic bases such as *p*-toluidine (Barton & Young, 1943) and *p*-bromoaniline (Laughland & Young, 1944) to give derivatives of the type $[ROSO_3]^-[R'NH_3]^+$. These derivatives are usually crystalline solids with a low solubility in water. In the present work 1- and 2-naphthylglucuronide were also found to form derivatives with organic bases, and by making use of this finding, 14·7–16·8% of the 1-naphthol and 18·0–22·5% of the 2-naphthol administered were recovered from the urine in the form of the *p*-toluidine salts of the corresponding glucuronides. The amounts of the naphthylsulphuric acids obtained from urine in the form of their organic base salts were quite small, and they represented only 0·4–0·6% of the 1-naphthol and 1·6–2·0% of the 2-naphthol administered. The amounts of the conjugated compounds obtained give little indication of the amounts excreted in the urine, however, because of the losses which occurred during the isolation processes. It seems probable, nevertheless, that the naphthylglucuronides were present in the urine in greater amounts than the naphthylsulphuric acids, for in a recent investigation (Young, Morrison & Billett, 1950) in which rats each received a single dose of 0·1 g. of 1- or 2-naphthol by subcutaneous injection, it was found that in the 3-day period after dosing the increase in urinary ethereal sulphate and glucuronide corresponded respectively to 21 and 53% of the 1-naphthol, and 20 and 56% of the 2-naphthol administered. It is noteworthy that Lesnik & Nencki (1886) reported that 1- and 2-naphthol are excreted mainly as glucuronides by the dog.

It was found that 1- and 2-naphthol were excreted by the dosed animals in the present work, for 2·4–2·8% of the 1-naphthol and 9·0–13·5% of the 2-naphthol were recovered unchanged from the urine. As with the conjugated naphthols, however, the object of the experiments was the separation of the compounds in pure form for identification purposes, and the quantities obtained do not give a true picture of the amounts of the compounds present in the urine.

SUMMARY

1. A study has been made of the metabolism of 1- and 2-naphthol following their administration to rats by subcutaneous injection.

2. The excretion of 1- and 2-naphthol in the urine of rats dosed with these compounds has been shown to occur.

3. 1-Naphthylsulphuric acid (as its *p*-bromoaniline salt) and 1-naphthylglucuronide (as its *p*-toluidine salt) have been isolated from the urine of rats dosed with 1-naphthol.

4. 2-Naphthylsulphuric acid and 2-naphthylglucuronide have been isolated in the form of their *p*-toluidine salts from the urine of rats dosed with 2-naphthol.

All the elementary micro-analyses reported above were carried out by Mr Michael Edson. An account of the work described in this paper formed part of a thesis presented to the University of Toronto (Berenbom, 1947).

REFERENCES

Barton, A. D. & Young, L. (1943). *J. Amer. chem. Soc.* **65**, 294.
Berenbom, M. (1947). Thesis, University of Toronto.
Feigenbaum, J. & Neuberg, C. A. (1941). *J. Amer. chem. Soc.* **63**, 3529.
Klyne, W. & Marrian, G. F. (1945). *Biochem. J.* **39**, xlv.
Klyne, W., Schachter, B. & Marrian, G. F. (1948). *Biochem. J.* **43**, 231.
Laughland, D. H. & Young, L. (1942). *Trans. roy. Soc. Can.* III, **36**, 166.
Laughland, D. H. & Young, L. (1944). *J. Amer. chem. Soc.* **66**, 657.
Lesnik, M. & Nencki, M. (1886). *Ber. dtsch. chem. Ges.* **19**, 1534.
Mauthner, J. (1881). *Jber. Fortschr. Tierchem.* **11**, 230.
Paterson, J. Y. F. & Klyne, W. (1948). *Biochem. J.* **43**, 614.
Young, L., Morrison, A. R. & Billett, F. S. (1950). Unpublished observations.

Chemical and Electrical Energy Relations for the Stomach

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The use of isolated acid-secreting gastric mucosa as experimental material makes it possible to measure concomitantly the rate of respiration (a process which makes energy available), and the rate of acid secretion (a process which requires energy). Moreover, the maximum energy that can be made available from respiration, and the minimum energy required to maintain the secretion, can be calculated. The efficiency of energy conversion can therefore be calculated by comparing the ratio of the rate of acid secretion to the rate of respiration found experimentally with the ratio calculated on the assumption that all the energy from respiration is available to do secretory work. This comparison indicates that the secretory process has a very high efficiency: it approaches 100%.

Similarly, the electrical work done by isolated gastric mucosa can be measured and compared with that available from exergonic processes in the tissue. In addition to this, the passage of an electric current through an acid-secreting gastric mucosa can increase or decrease the rate of acid secretion (Rehm, 1945; Rehm & Hokin, 1948; Crane, Davies & Longmuir, 1948*a*, *b*), and estimations can be made of the efficiency of these processes.

A part of this work was communicated to the Biochemical Society, on 24 September 1948 (Crane & Davies, 1948*a*, *b*).

EXPERIMENTAL

Saline media. The salt solutions were those described by Davies & Terner (1949). For manometric experiments the phosphate saline gassed with 100% O_2 was used; for the electrical experiments the bicarbonate saline, gassed with 5% CO_2 + 95% O_2, was used. Both saline solutions contained 0·2% (w/v) glucose.

Preparation of gastric mucosa. Frogs (*Rana temporaria temporaria* L.) were captured locally, housed in an outdoor froggery under natural conditions and provided with an excess of worms and insects for food (Bradford, Crane & Davies, 1950).

The gastric mucosa was washed and isolated immediately after the frog was pithed. For the manometric experiments it was then tied with silk at both ends and weighed (Davies, 1948*a*). For electrical experiments it was opened along the lesser curvature and mounted in a Perspex holder (Crane *et al.* 1948*a*).

Manometric experiments. The Q_{O_2} (μl./mg. dry wt./hr.) of the tied bags of frog gastric mucosa was measured at 25·0° with Warburg manometers in the usual way (Dixon, 1943). Conical cups (20–30 ml. capacity) were used and the CO_2 absorbed by alkali in the centre well. After a 20 min. equilibration period the pressure changes in the manometer were measured for 2–7 hr. Histamine was added from the side arm, when required, to a final concentration of 5×10^{-5} M, after the basal O_2 uptake had become steady. This was usually 1·5–2·5 hr. after the cups had been put into the bath.

At the end of the incubation the swollen bags of mucosa were removed, and the amounts of HCl produced were determined by weighing and by electrometric titration (Davies, 1948*a*; Davies & Longmuir, 1948).

Electrical experiments. The material, apparatus and methods used for the electrical experiments were similar to those previously described by Crane *et al.* (1948*a*, *b*). The Perspex holder containing the frog gastric mucosa was mounted between two chambers at 25·0°. The Q_{HCl} (μl./mg. dry wt./hr.) of the mucosa was estimated by measuring the changes in pH of the secretory solution *in situ*, and also by replacing the secretory solution and estimating its acid content either manometrically by the addition of a bicarbonate solution or by electrometric titration.

The potential difference (p.d.) across the mucosa was measured by a battery-operated Marconi pH meter and potentiometer Type TF 511 C. Saturated calomel electrodes and saturated KCl bridges with internal ground-glass joints, which dipped into the nutrient and secretory solutions, were

used. Current was passed through the mucosa by means of zinc-zinc acetate electrodes separated from the solutions bathing the mucosa by the agar layers previously described (Crane *et al.* 1948*a*). The resistance was obtained from the slope of the graph p.d. across mucosa against current through it, a correction being applied for the resistance of the solution.

RESULTS

Manometric experiments

Method of comparing the rates of respiration and acid secretion. When the oxygen uptake of a tied bag of gastric mucosa was measured, the amount of hydrochloric acid secreted in response to histamine could not be determined until the bag was opened at the end of the incubation period and the contents removed (Davies & Longmuir, 1948). The average Q_{HCl} after the addition of histamine could then be compared with the average increase in Q_{O_2} during the same period, and their ratio determined. As it was important that the mucosae should not be secreting acid spontaneously before the addition of histamine, these experiments were done in late autumn, winter and early spring, when isolated frog gastric mucosa does not secrete spontaneously (Bradford *et al.* 1950).

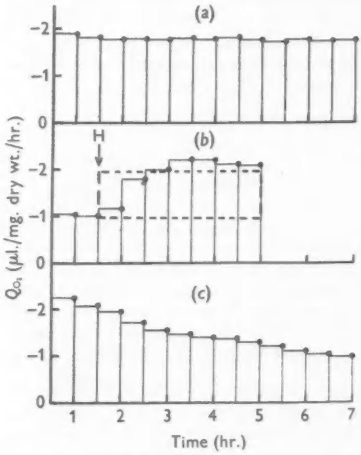

Fig. 1. Variation with time of Q_{O_2} of (*a*) normal unstimulated frog gastric mucosa, (*b*) mucosa which secreted acid after the addition of histamine at *H*, (*c*) abnormal mucosa with rapidly decreasing rate of respiration. The dotted lines in (*b*) show how the mean ΔQ_{O_2} (Table 2) was calculated.

Ulcers and perforations occurred regularly in tied bags of frog gastric mucosa which were secreting acid at high rates for 4–6 hr. in the absence of external supplies of carbon dioxide (Davies & Longmuir, 1948). In the present experiments the incubations were therefore usually continued for only 2–3 hr. after the addition of histamine, and any mucosa which perforated was rejected, since loss of secretion occurred through the hole formed in the bag.

Table 1. *Mean rates of acid secretion and respiration of isolated frog gastric mucosa*

Mean Q_{HCl} after histamine (μl./mg. dry wt./hr.)	Mean Q_{O_2} before histamine (μl./mg. dry wt./hr.)	Mean ΔQ_{O_2} after histamine (μl./mg. dry wt./hr.)
0·6	−1·4	−0·2
0·9	−1·7	−0·35
1·2	−2·2	−0·15
1·3	−1·5	−0·35
1·4	−0·9	−0·7
1·4	−1·9	−0·2
1·4	−1·8	−0·4
1·5	−1·6	−0·2
1·5	−2·0	−0·6
1·6	−2·0	−0·25
1·7	−2·5	−0·25
1·7	−1·4	−1·15
1·8	−2·5	−0·5
1·9	−2·0	−0·2
1·9	−2·1	−0·3
2·0	−3·0	−0·3
2·0	−2·2	−0·2
2·1	−2·9	−0·2
2·1	−2·6	−0·2
2·2	−2·2	−0·6
2·2	−1·7	−0·5
2·2	−1·9	−0·4
2·3	−2·0	−0·15
2·4	−1·0	−1·0
2·5	−3·2	−0·2
2·5	−1·5	−0·25
2·6	−3·0	−0·4
2·7	−1·9	−0·3
2·8	−2·4	−0·3
3·5	−2·6	−0·55
3·7	−2·6	−0·35
3·8	−2·0	−0·4
4·4	−2·7	−0·35
4·6	−2·1	−0·7

The average Q_{HCl} after the addition of histamine could be determined accurately; the average increase in the value of the Q_{O_2} (mean ΔQ_{O_2}) was subject to greater error, because the value the Q_{O_2} would have attained if the mucosa had not been stimulated was unknown. Fig. 1*a* shows the variation with time of the rate of respiration of a typical unstimulated mucosa. Fig. 1*b* is plotted for a mucosa which responded well to histamine. In calculating the mean ΔQ_{O_2} it was assumed that stimulated mucosae would have shown a steady behaviour similar to that of mucosa A if they had not been stimulated. Very occasionally (see Fig. 1*c*) the respiration rate of a resting mucosa was not maintained, but fell away rapidly. The respiration rate of a small proportion of the stimulated mucosae showed a similar fall; results for these mucosae were rejected.

Rates of respiration and acid secretion. Table 1 gives values of the mean Q_{HCl} after histamine, the mean Q_{O_2} before histamine and the mean ΔQ_{O_2} after histamine, for thirty-four isolated frog gastric mucosae. The ratio mean Q_{HCl}/mean $|Q_{O_2}|$ (the value of $|Q_{O_2}|$ is the positive number with the same numerical value as Q_{O_2}) after histamine had an average value of 0·9 for these mucosae. In Fig. 2 the mean Q_{HCl} after histamine is plotted against the mean ΔQ_{O_2} after histamine for these mucosae; the significance of the three lines through the origin is discussed later.

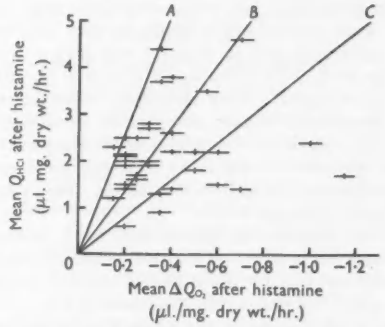

Fig. 2. Relation between mean Q_{HCl} and mean ΔQ_{O_2} after histamine for thirty-four isolated frog gastric mucosae. The horizontal and vertical lines represent the estimated errors in the mean ΔQ_{O_2} and Q_{HCl}. The significance of the lines *A–C* is discussed in the text.

Electrical experiments

The p.d. across isolated frog gastric mucosa decreases in magnitude when acid secretion commences, either spontaneously or after the addition of histamine (Crane *et al.* 1948*a*). The resistance of the mucosa, which is normally constant for current densities up to at least 0·4 mA./sq.cm. (Crane & Davies, 1950), was found in all of six experiments to increase at the onset of acid secretion (Crane *et al.* 1948*a*). These effects were reversed when acid secretion stopped.

Table 2 gives data for an average isolated frog gastric mucosa at 25·0° (Crane & Davies, 1948*b*). These data were obtained from a large number of separate experiments: it was not possible to measure all the variables for the same mucosa. A mean Q_{HCl} of 2·0 was chosen as representative (see Table 1, and also Bradford *et al.* 1950). The Q_{O_2} of −2·1 is an average value for fifty non-secreting isolated frog gastric mucosae (Crane *et al.* 1948*a*); the change of 0·3 in the mean Q_{O_2} during acid secretion is estimated from the results given in Table 1 and results for other mucosae (Davies, 1948*a*). The p.d. of −30 mV. before acid secretion is an average value for ninety-four mucosae and the p.d. decrease of 10 mV. is an average value for a Q_{HCl} of 2 (Crane *et al.* 1948*a*); the values for the resistance are taken from the same source. The latent electric power is the maximum electric power this average mucosa might be expected to produce (i.e. if it were in a circuit with zero external resistance); it is (p.d.)²/resistance, and the values given are for 1 mg. dry wt. (Crane *et al.* 1948*a*; Crane & Davies, 1950).

CALCULATIONS

Energy required for the production of gastric hydrochloric acid

The minimum free energy increase of the reactants (ΔF) which is required for the formation of hydrochloric acid in mammalian gastric mucosa has been calculated by several workers (Hill, 1931; Teorell, 1933; Hanke, 1937; Davenport & Fisher, 1940; Gray, 1943; Rehm, 1945). They all used the formula

$$\Delta F = nRT \log_e a_o/a_{pl},$$

where n is the number of g.mol. of ion secreted, R the gas constant, T the absolute temperature, and a_{pl} and a_o the molar activities of the ions in the plasma and the oxyntic cell secretion respectively. Since the primary gastric acid is approximately isotonic with blood (Babkin, 1950) the concentrations of the ions can be substituted for their activities.

The results do not agree in detail because the workers used different premises. Gray (1943) gives 1532 cal. as the minimum free energy required at 37° to form 1 l. oxyntic cell secretion containing 0·159M-H^+, 0·007M-K^+ and 0·166M-Cl^-. This is 9650 cal./g.mol. hydrochloric acid. For the acid secreted by frog oxyntic cells at 25° a similar calculation gives 8800 cal./g.mol. hydrochloric acid formed at 0·12M concentration. However, the effective minimum free energy required for secretion

Table 2. *Physical and metabolic data for an average isolated frog gastric mucosa*

(Temp., 25·0°; thickness of mucosa, 0·25 mm.; dry wt. in 1 sq.cm. of holder 9·5 mg.)

Variable	Value before HCl secretion	Value during HCl secretion	Change
Q_{HCl} (μl./mg. dry wt./hr.)	0	2·0	2·0
Q_{O_2} (μl./mg. dry wt./hr.)	−2·1	−2·4	−0·3
Potential difference (mV.)	−30	−20	10
Resistance of mucosa in 1 sq.cm. of holder (Ω-sq.cm.)	210	280	70
Latent electric power, i.e. (p.d.)²/resistance, for 1 mg. dry wt. (μW./mg. dry wt.)	0·45	0·15	−0·30

of acid may well be higher than the value calculated above on the basis of thermodynamic assumptions (isothermal, reversible reactions in ideal solutions).

It is generally accepted that the primary acid secretion is isotonic with blood collected at a distance from the stomach (Babkin, 1950; see however Linde, Teorell & Öbrink, 1947). This has been taken to imply that no osmotic work is done on the water of the secretion, since its mole fraction remains constant (Gray, 1943; Hollander, 1943). However, acid-secreting gastric mucosa replaces chloride ions from the blood quantitatively by bicarbonate ions (Davenport, 1946; Davies, 1948*a*). Therefore no ions are lost from the blood.

The volume of blood which is used for the production of 1 ml. of gastric juice depends on the rate of secretion; an average value of about 50 ml. can be calculated from the figures of Lim, Ni, Necheles & Chang (1929), Hanke (1937), Mann, Grindlay & Mann (1941) (dogs) and Teorell (1933) (cats). Isolated frog gastric mucosa, at 25° in a Warburg cup with 4·0 ml. nutrient solution, which secretes 0·1 ml. acid (a representative figure) must similarly concentrate the ions in the nutrient solution by 4·0/3·9. However, Ogston (1950) has shown that the osmotic work involved is only about 10 cal./mol. hydrochloric acid for both mammals and amphibia. It is of interest that if all the hydrogen ions come initially from water (discussed by Davies, 1951), about 2·9 ml. water must disappear for each litre of acid gastric juice secreted.

Energy available from metabolism within oxyntic cells, and values of $Q_{HCl}/|Q_{O_2}|$

Some thermodynamic relations for metabolic processes obtained by Ogston & Smithies (1948) have been applied to oxyntic cells by Davies & Ogston (1950). The maximum free energy available from the oxidation of glucose by atmospheric oxygen under physiological conditions of temperature, and glucose, oxygen and carbon dioxide concentrations is about 116,000 cal./mol. oxygen. The value 8800 cal./mol. hydrochloric acid, quoted above as the thermodynamic minimum free energy for the formation of frog gastric secretion, gives a maximum value for $Q_{HCl}/|Q_{O_2}|$ of about 13; the approximate value of 10,000 cal./mol. used earlier (Crane & Davies, 1948*a*) gives a ratio of 11·6.

The oxidation energy has been considered in two stages: first, the energy of oxidation between atmospheric oxygen and cytochrome *c* (ferri/ferro = 1), 52,000 cal./mol. oxygen, and secondly the rest of the energy, 64,000 cal./mol. oxygen; this division fits in well with the energy requirements of the previously described mechanisms 1 and 2 (Crane & Davies, 1948*a*; Davies & Ogston, 1950).

In mechanism 1 (Robertson & Wilkins, 1948; Conway & Brady, 1948; Crane & Davies, 1948*a*), the hydrogen ions come from the process

$$C_6H_{12}O_6 + 6O_2 + 18H_2O \rightarrow 6CO_2 + 24H^+ + 24OH^-;$$

this is the overall reaction for glucose oxidation, which gives a $Q_{HCl}/|Q_{O_2}|$ ratio of 4. The energy available from oxidations between oxygen and cytochrome *c* is ample to produce the gastric hydrochloric acid. In mechanism 2 (Crane *et al.* 1948*a*; Davies & Ogston, 1950) the energy available from the breakdown of each molecule of creatine phosphate produces two hydrogen ions. Reasons have been presented for believing that this mechanism, using the rest of the oxidation energy, allows values of $Q_{HCl}/|Q_{O_2}|$ up to 6·8.

If the increase in metabolism after histamine provides the power used for acid secretion, then the ratio (mean Q_{HCl} for the period of increased respiration after histamine/mean increase in Q_{O_2} for this period) is the same for the whole mucosa as for the secretory units of the oxyntic cells. In Fig. 2 the lines through the origin give the three $Q_{HCl}/\Delta|Q_{O_2}|$ ratios just discussed: *A* (13), *B* (6·8), *C* (4). Within experimental errors $Q_{HCl}/\Delta|Q_{O_2}|$ could be less than 13 for every mucosa represented in Fig. 2; less than 6·8 for two-thirds of them, and less than 4 for only one-third.

Energy available from electrical processes

The very high efficiences suggested by the results in Fig. 2, approaching 100% for one-third of the mucosa, make it necessary to consider whether any energy is available for acid secretion from the basal metabolism of the mucosa. The latent electric power of the average mucosa (Table 2) was 0·30 μW./mg. dry wt. less during secretion than before, and it seems possible that this power is used for secretory work. Since 1 cal./sec. = 4·2 W., the power required for a Q_{HCl} of 2 in frog gastric mucosa is 0·9 μW./mg. dry wt. (using the figure 8800 cal./mol. HCl), so that in this case an appreciable fraction, one-third, of the energy needed for hydrochloric acid secretion would come from basal metabolic energy. It is clear, however, that the whole of the energy needed for high rates of hydrochloric acid secretion could not come from this source: the total latent electric power of a mucosa with a p.d. of even 50 mV. could not support a Q_{HCl} greater than 2·5, and values of 8 or more have been observed.

Using the assumptions on which lines *A* and *B* (Fig. 2) are drawn, the average increase in $|Q_{O_2}|$ of 0·3 on secretion would provide 1·8 and 0·9 μW./mg. dry wt. respectively. If *A* is correct, the increase in Q_{O_2} can provide all the energy needed for hydrochloric acid secretion in the average mucosa if the efficiency of utilization of the energy from increased respiration is 0·9/1·8, or 50%. On the other hand, if

B is correct the efficiency must be 0·9/0·9, or 100 %. But if that part of the metabolic energy represented by the change in the latent electric power is also available for secretion, the efficiency of utilization of the increase in metabolic energy need be only 0·6/1·8, or 33 % for *A* or 0·6/0·9 or 67 % for *B*.

DISCUSSION

Rates of respiration and secretion in frog gastric mucosa

In calculating efficiencies, the comparison of the mean Q_{HCl} after histamine stimulation with the increase in the rate of oxygen uptake after stimulation is based on the following four assumptions: (1) the acid collected at the end of the experiments is the whole of the acid secreted; (2) the whole of the increase in respiration of the mucosa following the addition of histamine supplies energy used by the oxyntic cells for the secretion of hydrochloric acid; (3) only aerobic reactions lead to the production of hydrochloric acid; (4) the basal respiration of the whole mucosa is needed to maintain the general integrity of the tissue and does not provide either hydrogen ions or energy for acid secretion.

Assumption (1) is based on the following results. Teorell (1933, 1939, 1947) found that hydrogen ions can diffuse through mammalian gastric mucosa, and Terner (1949) found that back-diffusion of hydrogen ions can also occur in frog and toad gastric mucosa, but that it is negligible unless the pH is less than about 2. In the present series of experiments the pH of the total gastric contents was always above 2 (cf. Davies & Longmuir, 1948) so the loss of hydrogen ions from this cause would be insignificant (cf. Davies & Ogston, 1950).

Evidence in favour of assumption (2) is provided by the fact that the respiratory and secretory activity of only the oxyntic cells respond to histamine; other gastric cells are not affected (Davies, 1948*a*; Babkin, 1950).

Assumption (3) is probably correct because hydrochloric acid secretion stops when oxygen is removed or its utilization inhibited in both isolated tissue (Ivy, 1943; Davies, 1948*a*, 1950; Crane, Davies & Longmuir, 1946; Crane *et al.* 1948*a*) and in the intact stomach (Rehm & Hokin, 1947; Rice & Ross, 1947). In anaerobiosis the p.d. across the mucosa is abolished, and the stomach ferments glucose to lactic acid. It is of interest that oxyntic cells can secrete continuously without fatigue (Hollander, 1943; Babkin, 1950), in contrast to muscle which can do work anaerobically, develop an oxygen debt, and become fatigued.

Assumption (4) presents more difficulties; if it is true, the thermodynamic efficiency of hydrochloric acid production must be remarkably high: it was 50 % or more for about two-thirds of the mucosae used and 100 % for some, within the experimental error (Fig. 2). Moreover, it would exclude either of the mechanisms 1 and 2 (lines *C* and *B*) acting alone, since either would give an efficiency greater than 100 % for many mucosae.

It may be, however, that part of the energy or some of the hydrogen ions from the unstimulated respiration can be utilized for acid secretion. In this case the experimental results do not exclude mechanism 2 (*B*) or even mechanism 1 (*C*) alone, since the mean Q_{HCl} of the mucosa for the whole experiment was never greater than the mean Q_{O_2}. This result is expected when phosphate saline gassed with 100 % oxygen is used and carbon dioxide is absorbed by alkali in the centre well of the Warburg cup (Davies & Longmuir, 1948), because there are then no external supplies of carbon dioxide available to the tissue to maintain the acid/base balance within the oxyntic cells. However, during the initial unstimulated respiration there is built up a steady-state concentration of carbon dioxide in the liquid and gas spaces of the manometer. After the onset of secretion some of this carbon dioxide can be used by the mucosa, so a high rate of acid secretion (and of carbon dioxide uptake) can be maintained for a short period, resulting in a Q_{HCl} higher than the stimulated Q_{O_2} (Table 1).

In order to assess the part played in acid secretion by the resting respiration of the whole mucosa, which can be measured, it is necessary to know the fraction of this respiration due to the oxyntic cells. Unfortunately this cannot be determined with techniques at present available. The cells comprise about 10 % or less of the volume of gastric mucosa (Davies & Roughton, 1948), but Engström & Glick (1950) have shown recently that the ratio dry weight/wet weight for dog oxyntic cells is only 0·1, which is half the average for the whole mucosa; their dry weight is therefore only 5 % of that of the whole mucosa. This means that only a small fraction of the resting metabolism of the mucosa occurs in the oxyntic cells. The resting Q_{O_2} of the corpus region of frog gastric mucosa, containing oxyntic cells, which constitute only a small fraction of its dry weight, is however somewhat higher than that of the pyloric antrum (Davies, 1948*a*), which contains none. Therefore the resting Q_{O_2} of these cells is probably greater than the average Q_{O_2} of the whole mucosa.

Some confirmation of the results in Fig. 2 has been published by Teorell (1949*a*). He measured the rate of acid secretion of a sheet of isolated frog gastric mucosa stimulated with histamine in a bicarbonate saline gassed with 5 % CO_2 + 95 % O_2 (Teorell & Wersäll, 1945), and then transferred the membrane to a Warburg cup and measured the Q_{O_2} in phosphate saline. This method has the advantage that throughout the experiments carbon dioxide is either available externally, or is continuously reformed from bi-

carbonate (Davies & Longmuir, 1948). Thus no damage occurs, even at high rates of acid secretion. A disadvantage is that the rates of acid secretion and of respiration are measured for consecutive periods, when conditions may not be the same. The Q_{HCl} and Q_{O_2} were measured for four to six mucosae at 15, 20, 25, 28 and 33°. The results showed that '$Q_{HCl}/|Q_{O_2}|$ is of the order of 2·2 (probable variation 1·1–4·4)'. It is important to realize that the total $|Q_{O_2}|$ values were used; the comparable average ratio in the present experiments is 0·9. Teorell (1949*a*) did not determine the increase in $|Q_{O_2}|$ on acid secretion, so his results are not directly comparable with those in Fig. 2. However, they emphasize the remarkably high efficiency of acid-secreting gastric mucosa.

Electrical energies in amphibian and mammalian gastric mucosa

It is shown above that part, but not the whole, of the energy needed for acid secretion in isolated frog gastric mucosa could come from the energy which, in the resting mucosa, is available for doing external electrical work. There is no direct evidence that this is so, but it is consistent with experimental results, and is reasonable on theoretical grounds (Crane *et al.* 1948*a*, *b*; Davies & Ogston, 1950). The two main experimental results, the decrease in p.d. across the mucosa (and therefore in the electric power available externally) at the onset of acid secretion, and the increase or decrease in the rate of acid secretion by the passage of current through mucosae already secreting acid, according to the direction of the current, were also observed in live dogs (Rehm, 1944, 1945).

Teorell (1949*a*) wrote 'although a covariation between secretion rate and potential could be traced in some single experiments, it was not possible, in our material, to find such a pronounced general relationship between potential and H^+ production, as has recently been argued by Rehm (1944, 1945) and Crane, Davies & Longmuir (1948*a*)'. However, it appears (Teorell, 1949*b*), that in many of his experiments acid secretion was occurring spontaneously before the p.d. changes following the addition of histamine were measured. Both Rehm (1944, 1945, 1950) and the present writers have found that the major change in p.d. takes place at the onset of secretion, and that further increases in the secretory rate normally have smaller effects. This probably accounts for the apparent discrepancy between the results.

Rehm (1944, 1950) and Rehm & Hokin (1947) have pointed out that in dog stomach the rate of acid secretion increases from low to high rates whilst the p.d. across the stomach wall varies by only a few millivolts; the extra electrical energy available is quite insufficient to account for the increased rate of secretion. The increase in the rate of respiration during acid secretion in mammalian stomach, which would supply energy to maintain all or part of the secretion, has not yet been determined. Further discussion on the electrical energy relations will be postponed until this and other information is available.

Rates of respiration and secretion in mammalian gastric mucosa

It is technically very difficult to measure the oxygen consumption of a mammalian stomach which is actively secreting acid, and rates of secretion measured at the same time as the Q_{O_2} have been much lower than those found in animals not subject to the severe operational trauma needed for such experiments. However, the maximum rates of acid secretion for men and dogs which have been found (Davies, 1948*a*, *b*) are up to 100 μl./mg. dry wt./hr. for the whole gastric mucosa; the Q_{HCl} of the oxyntic cells must therefore have been at least 2000. This rate of activity is remarkably high compared with metabolic quotients for other mammalian cells (cf. Krebs & Johnson, 1948).

Even on the assumption of 100 % efficiency in the secretion of 0·16 M-hydrochloric acid, the Q_{O_2} of the oxyntic cells must be -170 for ratio *A*, -200 for *B* (mechanism 2) and -500 for *C* (mechanism 1). These figures are about twice those given by Crane & Davies (1948*a*), which were calculated before the results of Engström & Glick (1950) were published (see p. 173). They must be exceeded if the cells are not 100 % efficient.

Since $|Q_{O_2}|$ values for most mammalian tissues are less than 20, maximally secreting oxyntic cells must be amongst the most active mammalian cells. It is, however, unlikely that values of $|Q_{O_2}|$ as high as 500 occur in any mammalian cells; this supports the view that mechanism 1 above is quantitatively inadequate, and that most of the metabolic energy of oxyntic cells is canalized into making the hydrochloric acid, by mechanisms 1 and 2 acting together as discussed by Davies & Ogston (1950).

Although the oxyntic cells must have such a high rate of respiration, the Q_{O_2} of the gastric mucosa is probably within the range of Q_{O_2} values for other tissues, even when it is secreting acid: the increase in Q_{O_2} of the gastric mucosa due to maximal acid secretion would be only one-twentieth of the values quoted above. It is of interest that most oxyntic cells are separated from each other, with their bases on the outside of the gastric tubules very close to the capillary network. This makes possible the high rates of exchange between these cells and the blood, of oxygen, carbon dioxide, water and substrates, which occur during high rates of acid secretion. A system of highly active cells embedded in a less active structure may also occur in tissues with high average rates of metabolism such as kidney and retina.

SUMMARY

1. Measurements have been made of the mean Q_{O_2} of isolated frog gastric mucosa before histamine stimulation and of the mean Q_{HCl} and Q_{O_2} during the secretory period.

2. Figures are given for the changes on secretion of the p.d., resistance and latent electric power of a representative mucosa, with corresponding Q_{O_2} and Q_{HCl} values.

3. The energy required for the production of gastric hydrochloric acid is compared with the energy available from metabolism. If the increase in the rate of respiration of the mucosa provides the power for secretion, the efficiency of the secretory process is very high, approaching 100 %. A quantitative discussion is given of the possibility that energy is also available for secretion from the basal metabolic energy, either as a result of the change in latent electric power or otherwise.

4. It is shown that mammalian oxyntic cells are among the most active cells in the body.

REFERENCES

Babkin, B. P. (1950). *Secretory Mechanism of the Digestive Glands*. New York: Hoeber.

Bradford, N. M., Crane, E. E. & Davies, R. E. (1950). *Proc. zool. Soc. Lond.* **120**, 471.

Conway, E. J. & Brady, T. G. (1948). *Nature, Lond.*, **162**, 457.

Crane, E. E. & Davies, R. E. (1948*a*). *Biochem. J.* **43**, xliii.

Crane, E. E. & Davies, R. E. (1948*b*). *Biochem. J.* **43**, xliii.

Crane, E. E. & Davies, R. E. (1950). *Trans. Faraday Soc.* **46**, 598.

Crane, E. E., Davies, R. E. & Longmuir, N. M. (1946). *Biochem. J.* **40**, xxxvi.

Crane, E. E., Davies, R. E. & Longmuir, N. M. (1948*a*). *Biochem. J.* **43**, 321.

Crane, E. E., Davies, R. E. & Longmuir, N. M. (1948*b*). *Biochem. J.* **43**, 336.

Davenport, H. W. (1946). *Physiol. Rev.* **26**, 560.

Davenport, H. W. & Fisher, R. B. (1940). *Amer. J. Physiol.* **131**, 165.

Davies, R. E. (1948*a*). *Biochem. J.* **42**, 609.

Davies, R. E. (1948*b*). The mechanism of hydrochloric acid production by the stomach. Ph.D. Thesis, University of Sheffield.

Davies, R. E. (1951). *Biol. Rev.* **26**, 87.

Davies, R. E. & Longmuir, N. M. (1948). *Biochem. J.* **42**, 621.

Davies, R. E. & Ogston, A. G. (1950). *Biochem. J.* **46**, 324.

Davies, R. E. & Roughton, F. J. W. (1948). *Biochem. J.* **42**, 618.

Davies, R. E. & Terner, C. (1949). *Biochem. J.* **44**, 377.

Dixon, M. (1943). *Manometric Methods*, 2nd ed. Cambridge University Press.

Engström, A. & Glick, D. (1950). *Science*, **111**, 379.

Gray, J. S. (1943). *Gastroenterologia*, **1**, 390.

Hanke, M. E. (1937). *Science*, **85**, 54.

Hill, A. V. (1931). *Adventures in Biophysics*. London: Milford.

Hollander, F. (1943). *Gastroenterology*, **1**, 401.

Ivy, H. B. (1943). Quoted by Gray (1943).

Krebs, H. A. & Johnson, W. A. (1948). *Tabul. biol., Berl.*, **19**, 100.

Lim, R. K. S., Ni, T. G., Necheles, H. & Chang H. C. (1929). *Chin. J. Physiol.* **3**, 123.

Linde, S., Teorell, T. & Öbrink, K. J. (1947). *Acta physiol. scand.* **14**, 220.

Mann, F. D., Grindlay, J. H. & Mann, F. C. (1941). *Amer. J. digest. Dis.* **8**, 451.

Ogston, A. G. (1950). Private communication.

Ogston, A. G. & Smithies, O. (1948). *Physiol. Rev.* **28**, 283.

Rehm, W. S. (1944). *Amer. J. Physiol.* **141**, 537.

Rehm, W. S. (1945). *Amer. J. Physiol.* **144**, 115.

Rehm, W. S. (1950). *Gastroenterology*, **14**, 401.

Rehm, W. S. & Hokin, L. E. (1947). *Amer. J. Physiol.* **149**, 162.

Rehm, W. S. & Hokin, L. E. (1948). *Amer. J. Physiol.* **154**, 148.

Rice, H. V. & Ross, R. T. (1947). *Amer. J. Physiol.* **149**, 77.

Robertson, R. N. & Wilkins, M. J. (1948). *Nature, Lond.*, **161**, 101.

Teorell, T. (1933). *Skand. Arch. Physiol.* **66**, 225.

Teorell, T. (1939). *J. gen. Physiol.* **23**, 263.

Teorell, T. (1947). *Gastroenterology*, **9**, 425.

Teorell, T. (1949*a*). *Experientia*, **5**, 409.

Teorell, T. (1949*b*). Private communication.

Teorell, T. & Wersäll, R. (1945). *Acta physiol. scand.* **10**, 243.

Terner, C. (1949). *Biochem. J.* **45**, 150.

The Uptake and Loss of Phosphate by Frog Muscle

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Phosphate, which has been labelled by the admixture of a proportion of radioactive phosphorus combined as phosphate, has been the most exploited radical containing an artificially produced isotope in biological studies. In connexion with muscle metabolism alone there are many investigations on the rate of incorporation of labelled phosphate into the various organic phospho-compounds found associated with the muscle cell. Attempts have been made to relate the rate of turnover with muscular activity, and with the presence of metabolically active substances, such as insulin. Reviews of these results have been made by Wood (1948) and Sacks (1948). Insufficient attention appears to have been paid to the primary act of absorption of the phosphate. Two alternative theories exist: (*a*) that it enters the cell as such, which is implied in Krogh's (1946) calculation of cell permeabilities to phosphate from Hevesy & Rebbe's (1940) results; and (*b*) that it enters into combination at the surface of the cell, possibly forming hexosephosphate in an adsorbed state (Sacks, 1948). Rothstein & Meier (1948) have shown that intact yeast cells have the property of splitting a number of phosphates, and they consider that the enzymes responsible are located on the cell surface rather than in the interior. This, and Sacks's conclusions, suggest that attention has to be paid to the possibility of reactions in an adsorbed layer, rather than in the interior of the cell.

Before radioactive phosphorus was available Eggleton (1933), by first soaking muscles in solutions containing known phosphate concentrations, and then either analysing the muscles or the solutions, found that the proportion of the muscle water into which phosphate penetrated was 0·24, which, taking the muscle as 80 % water, is 0·19 by volume of the whole muscle. This agrees well with the fractional extracellular volume found in other ways, e.g. by radioactive sodium in the experiments of Harris & Burn (1949). Eggleton concluded that the penetration of phosphate into the cells was negligible, even after 24 hr.

Hahn, Hevesy & Rebbe (1939) used radioactive phosphate for experiments made *in vivo*, and found that the uptake of the ^{32}P exceeded that expected for the extracellular spaces, so they supposed that penetration of the cells had taken place. Later work by Hevesy and his collaborators (1940) showed that radioactive phosphorus appeared both in the inorganic and the organic phosphate fractions which could be extracted from the cells.

The reason for working in vitro. The kinetics of phosphate absorption has not been investigated *in vitro*, and since the uptake commonly measured has hitherto been from a varying concentration of labelled phosphate in plasma following an injection, this phosphate could well have included much organically bound labelled phosphate at the end of the experimental period. Govaerts (1948) has found that freshly injected labelled phosphate does not resemble the circulating phosphate of blood; evidently any difference in state of combination interferes with a quantitative study of the uptake in terms of blood inorganic phosphate. To avoid a variable concentration of inorganic labelled phosphate in the blood stream and the possibility of interference by organically bound labelled phosphate formed during the experiment one can use isolated muscles *in vitro*. In this case the transfer from the medium to the cells has to take place through the extra cellular spaces, and it is important to use thin muscles (Harris & Burn, 1949). This consideration does not arise *in vivo* because of the capillaries.

The removal of phosphate in extracellular fluid. When it is desired to measure the quantity of labelled phosphate which has become incorporated in a specimen following exposure to a solution containing labelled phosphate (denoted by *P) it is necessary to distinguish that part of the *P present in the extra cellular space from that part associated with the cells. For this purpose Kalckar, Dehlinger & Mehler (1944), before making analyses, perfused the muscles taken from rabbits which had been previously injected with *P, whilst Furchgott & Shorr (1943), in a study of absorption of *P by cat heart slices, washed the specimens for 30 min. before analysis. The washing or perfusion procedure certainly leads to a constant residual quantity of *P, but clearly it would be possible for any readily removable cellular phosphate to be taken out along with the extracellular phosphate. This would only be made apparent if the extracellular volume were known, and the quantity of readily removable phosphate was compared with the quantity calculated from the *P in the medium and the extracellular space. In Furchgott & Shorr's material the

calculated extracellular space was about 25 %, which is said (Wood, 1948) to agree with the value found in other ways, in which case it is fair to conclude that no readily removable cellular fraction was present in or on the cells. Manery & Bale (1941) injected a solution containing *P into rats, and after 20 min. dissected out the gastrocnemii. The *P uptake corresponded to that expected in 12–14 % extracellular space, which is a reasonable value; it appears that under these conditions little uptake by the cells had occurred, which is surprising in the light of experiments we have made. These show *P attached to the cells (Pl. 1 *C*) following the injection of *P into a rat. The frog muscles used in the present study took up a considerable excess of *P over the amount calculated for the extracellular space, knowing the concentration of *P in the medium. Some of the excess was comparatively readily lost to a solution free from *P, but a considerable part was firmly held in some way by the muscle.

The points to be elucidated include the questions (*a*) how is the amount of *P held related to the concentration in the medium; (*b*) where is the *P held; and (*c*) in what form is the *P held?

EXPERIMENTAL

For most experiments frog sartorii were used, as they can be easily dissected free from other tissue without damaging the fibres.

The treatment of the radioactive phosphate will be described later. For a 'soaking-in' experiment, that is one in which the uptake of *P from a solution was to be followed, the muscle was immersed in a solution containing ^{32}P combined as phosphate together with a known concentration of non-radioactive phosphate, made by adding 0·1 M-phosphate to the usual concentrations of NaCl and KCl found in frog Ringer solution. Ca was sometimes omitted from the media in order to avoid precipitation of calcium phosphate. There was a considerable spontaneous twitching set up in muscles in some of these media. At suitable intervals the muscle was removed, washed for 15 sec. in a solution free from *P and placed on a clean glass cover slip at a suitable distance beneath a Geiger counter for assay of the radioactivity. This treatment removes a certain fraction of the extracellular *P, and the quantity is imperfectly reproduced at successive counts, but when the total uptake exceeds that corresponding to the extracellular *P the error is not serious. It was not possible to make use of a frame for maintaining the muscle in a standard position because the adsorption of radioactive phosphate on to the frame proves so troublesome.

For soaking-out experiments, in which a muscle which had previously been made radioactive was treated with an inactive solution, a frame could be used, but it was advantageous to prevent adsorption from the muscle on to the frame during the first 15 min. of soaking-out by the temporary interposition of a thin polythene sheet between frame and muscle; this sheet was removed after 15 min. by which time little *P moved from the muscle on to the adjacent solid.

At the end of the experiment the muscle was treated in either of two ways. Alternatively it was dissolved in a few drops of acid, and a sample of the solution used for comparison of the radioactivity with that of the radioactive solution used for the treatment of the muscle, or the muscle was frozen in solid CO_2, pulverized, and extracted with ice-cold 5 % (w/v) aqueous trichloroacetic acid in order to analyse for phospho-compounds. This was carried out by adding magnesium ammonium chloride and ammonia, to precipitate the inorganic phosphate, followed by the addition of acid and ammonium molybdate to the supernatant liquid. After standing for 1 hr. in order to hydrolyse phosphocreatine, the liquid was again made alkaline to precipitate the phosphate derived from phosphocreatine, and the supernatant liquid was again made acid and boiled for 7 min. in order to obtain a precipitate of ammonium phosphomolybdate derived from what will be called the pyrophosphate fraction. The debris from the muscle after trichloroacetic acid extraction was also dissolved in HNO_3 for assay of radioactivity and analysis for phosphate. The phosphate content of the various fractions was estimated colorimetrically using the Fiske-Subbarow method, with amidol reducer; and the radioactivities were assayed using the liquid counter.

RESULTS

The radioactive phosphate. Before starting the earlier experiments the solution of radioactive phosphate was mixed with a small amount of orthophosphate and the mixture boiled with the addition of a little nitric acid, such that the concentration of nitric acid was initially 5–10 %. A trace of this mixture was added to Ringer solution and the phosphate concentration made up to the desired value with orthophosphate. The solution was then used to treat a muscle.

It was sometimes found that the uptake of radioactivity by the muscle was as much as ten times the usual, and the time course of the uptake was most erratic. Strong adsorption of the radioactivity on to the glassware was noticeable, even in the presence of high concentrations (up to 0·1 M) of ordinary phosphate. This suggested that part of the radioactivity might be in a form different chemically from orthophosphate. The suppliers of the radioactive phosphate have in fact agreed that some 3 % of the ^{32}P might have been so combined. Fig. 1, curves *A* and *B*, illustrates the uptake of ^{32}P from solutions containing radioactive phosphate which had been acid boiled, and made up with Ringer solution so that a concentration of 200 μg. P as phosphate per ml. was present.

The uptake has been expressed as the ratio of radioactivity per g. of muscle to radioactivity per ml. solution; the extracellular spaces alone, if nothing was lost in the 15 sec. wash, would give a ratio of 0·13–0·2, this range being based on work with radioactive sodium (Harris & Burn, 1949). Clearly the uptake of ^{32}P is much greater than corresponds to extracellular solution. On changing to a non-radioactive solution there was a rapid loss of activity for

a short time, but the ratio remained high even after an hour of washing, indicating that much ^{32}P had become firmly attached to the muscle. The quantity of phosphate so attached could be calculated from the relation between the radioactivity of the soaking medium and the chemical concentration of phosphate, and it proved to be very much in excess of that expected if penetration of the muscle cells was involved. When, however, the solution of radioactive phosphorus, mixed with sufficient P to give finally 60 or 200 μg./ml., was evaporated to dryness with nitric acid and taken up in Ringer solution the results obtained were different, the uptake and loss being exemplified by curves *C*, *D* and *E*. There seems no doubt then that boiling with rather dilute nitric acid is insufficient to ensure that all the ^{32}P is converted to orthophosphate, though evaporation to dryness with the acid does appear to be more effective.

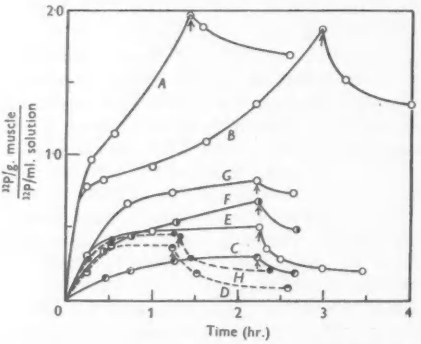


Fig. 1. Curves showing the uptake of radioactive phosphorus compounds from mixtures of various phosphates with the preparation containing ^{32}P, and also the loss of activity when the specimen was transferred (indicated by arrow) to a non-radioactive solution. *A*, uptake from ^{32}P which had been boiled with acid, the solution contained 200 μg. P/ml. as orthophosphate. *B*, as *A*. *C*, uptake from ^{32}P which had been evaporated to dryness with acid. The solution contained 61 μg. P/ml. as orthophosphate. *D* as *C*, but with 200 μg. P/ml. *E* as *C*. *F*, uptake from labelled phosphate which had been boiled with acid, the solution contained 200 μg. P/ml. as hexametaphosphate. *G*, as *F*, but with 200 μg. P/ml. as tripolyphosphate. *H*, uptake from ^{32}P which had been boiled with acid. The solution contained 66 μg. P/ml. each as orthophosphate, tripolyphosphate and hexametaphosphate.

A few experiments were made to test the effectiveness of other phosphorus compounds in reducing the uptake of the muscle by competition. Mixtures of the radioactive phosphate which had only been boiled with acid, and a trace of orthophosphate as carrier, were made with metaphosphate, pyrophosphate, hexametaphosphate, and tripolyphosphate, all with the equivalent of 200 μg. P/ml., in Ringer solution. The uptake by muscles from the first two was as much as, or more than, that from the 200 μg. P/ml. orthophosphate, but the two latter led to a smaller uptake than the others (Fig. 1, curves *F* and *G*). Finally, a mixture of 66 μg. P/ml. each of orthophosphate, hexametaphosphate and triphosphate was tried (curve *H*) and the behaviour of the muscle in this resembled that in the acid evaporated ^{32}P + orthophosphate solution (curves *C*, *D* and *E*). This suggests that the sites on to which is adsorbed the unknown ^{32}P compound, present in the solution which has only been acid boiled, can be saturated by complex phosphates.

The evaporation to dryness with acid of the ^{32}P solution evidently removes part, or all, of the interfering compounds, but a reliable method of treatment and testing has yet to be evolved. Possibly an ion-exchange resin, previously saturated with orthophosphate, will prove to be the best agent for treating the ^{32}P solution, for the interfering compounds are readily adsorbed.

The site at which the radioactive phosphate is held. The ready adsorbability on all surfaces of the radioactive phosphorus compounds made it desirable to see whether phosphate was adsorbed on muscle too. Radioautographs showed that this was so. This in itself would not be of fundamental biological importance if it were attributable only to traces of radioactive impurities in the ^{32}P preparation, except in so far as it may apply to other researches employing ^{32}P. However, use of a ^{32}P solution which has been evaporated to dryness with acid also leads to the same result.

Radioautographs of muscles prepared either by soaking to constant radioactivity in a solution containing ^{32}P or by injection of ^{32}P into the frog, followed by a wash of 15 min. in a plain solution to remove extracellular ^{32}P, reveal the presence of a surface layer containing ^{32}P. The muscles were either fixed (Pl. 1*C*, *D*) and cut in wax, or frozen (Pl. 1*A*, *B*) in solid carbon dioxide and cut on a freezing microtome, in which case the sections were pressed on to a slide with filter paper, and exposed to formaldehyde vapour for 20 min. Kodak stripping film was floated on to the sections and dried. In this manner a close approximation of film and section was obtained. The film was developed after 14 days' exposure, giving an autograph in contact with the original section. The section was then lightly stained with eosin, and the whole preparation photographed.

The black granules of silver in the parts made sensitive to reduction by the β-particles of the ^{32}P can be seen at the edges of the fibres. In the frozen sections the cutting necessarily caused damage to the fibres, and distortion, but the concentration of radioactive phosphorus at the edges is maintained despite the mechanical disturbance. The fact that

2–4 μ. separates the silver in the emulsion from the section makes it impossible to have both in focus at the same time. A plate of a longitudinal section of

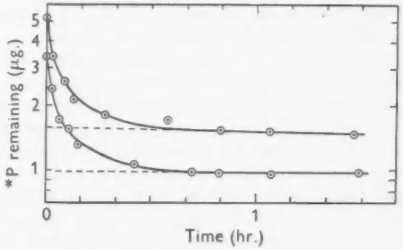


Fig. 2*a*. Curves exemplifying the loss of the *P from muscles at 18° when only extracellular and fixed phosphate are present. (Pretreated in 200 μg. *P/ml. Ringer solution.) One side exposed. 0·8 mm. thick.

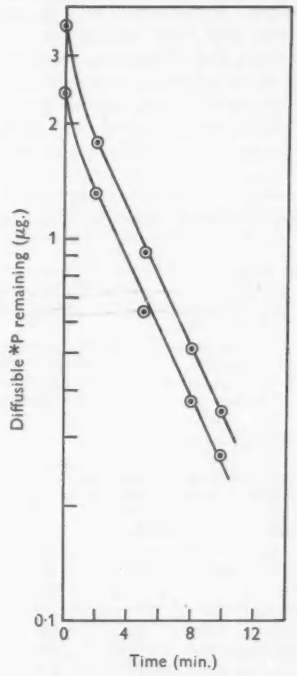


Fig. 2*b*. Curves derived from those of Fig. 2*a* by subtraction of the fixed *P from the total *P.

rat muscle, taken after injection of ^{32}P into the live rat has been included to illustrate the generality of the behaviour.

Study of the radioautographs led to the conclusion that the majority of the radioactive phosphorus remaining after 15 min. washing is held at the cell surfaces, though there is evidence that the nuclei, which make up a very small proportion of the total volume, also contain it.

The uptake and loss of phosphate. The more informative experiments were made by soaking the specimens in a solution containing ^{32}P and phosphate, for a given time, and then following the loss of activity to a plain solution. The curves *C*, *D*, *E* in Fig. 1 indicate the time scale of both processes. After 0·5–2 hr. uptake ceases, presumably because the formation of an adsorbed layer is complete.

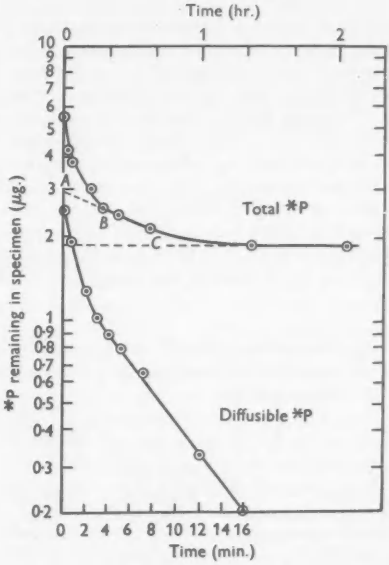


Fig. 3. Upper curve: the loss of *P from a muscle at 18° when there appears to be an additional fraction which is lost more slowly than the extracellular *P. (Pretreated in 100 μg. *P/ml. Ringer solution.) One side exposed. 0·8 mm. thick. Lower curve: the result of subtracting values along *A*–*B* from the total *P and replotting on an expanded time scale.

If the loss of the ^{32}P-labelled phosphate to Ringer solution is observed in more detail than in the experiments of Fig. 1, curves exemplified by Figs. 2*a* and 3 are obtained. In the examples in Fig. 2*a* the part of the radioactivity which is lost corresponds well with that expected for the ^{32}P in the extracellular spaces, and by subtracting the residual activity from the activity at various times, a new curve (Fig. 2*b*) can be constructed, which may be presumed to correspond to the diffusion of extracellular phosphate into the medium. The extracellular space estimated from the amount of rapidly

lost phosphate was $0{\cdot}20 \pm 0{\cdot}05$ (mean of 26) of the muscle volume. The diffusion of phosphate ions proceeded somewhat more slowly than that of sodium ions. The method of calculation of extracellular space and of the effective diffusion constant has been described previously (Harris & Burn, 1949).

Curves like those of Fig. 3 are more equivocal, for it becomes difficult to distinguish extracellular phosphate from that derived from the cells. By projecting the part of the curve, *B–C*, to meet the axis at *A*, and taking differences as before, one arrives at the lower curve of Fig. 3, from which, just as from Fig. 2*b*, the extracellular space, and the diffusion constant of the phosphate ion can be evaluated. In some experiments no separation was possible. It is not at all unreasonable that a part of the adsorbed phosphate should be readily lost, and so liable to be confused with extracellular phosphate. This, if it takes place, gives an erroneously high value for the extracellular space in experiments in which all the rapidly lost phosphate is attributed to extracellular material. The slow rate at which phosphate eventually leaves the cells (as little as 10% loss overnight) is remarkable, but is paralleled by the slow loss of ^{32}P from erythrocytes which allows them to be used in the estimation of blood volume.

An attempt was made to study the amount of phosphate taken up by the muscle cells as a function of treatment, but the results were very scattered. There are a number of reasons why this should be so, amongst them being the doubt about the complete removal of traces of readily adsorbed ^{32}P compounds, and the variation of mean specific surface of the specimens. The effect of the trace compounds upon the manner in which the radioactivity becomes distributed between the various phosphate compounds was studied by comparing the analyses of muscles *B* and *E*, Fig. 1: *B* was treated with solution containing ^{32}P which had only been boiled with acid, whereas *E* was treated with a solution which had been evaporated to dryness with acid. Table 1 shows that the additional ^{32}P which *B* acquired remained attached to the trichloroacetic acid-insoluble fraction.

Table 2 shows the results of analyses of a number of muscles which had been soaked in mixtures of the ^{32}P and phosphate, with Ringer solution. The pH was 7·4 and the temperature 18°. Before analysis the specimens had been washed in an inactive solution having a low phosphate concentration until their residual radioactivity was substantially constant. Unfortunately these experiments were made using ^{32}P which had only been acid boiled so the debris results may be too high in some cases.

Table 1. *Comparison of the ^{32}P contents of the phosphate fractions derived from muscles soaked in ^{32}P preparations which had alternatively been boiled with acid, or evaporated to dryness with acid, before addition to the soaking solution*

(Muscles *B* and *E* of Fig. 1.)

	Muscle *B*		Muscle *E*	
	P content	^{32}P	P content	^{32}P
Solution used	200 μg./ml.	34,500 counts/min./ml.	61 μg./ml.	22,300 counts/min./ml.
Acid-soluble inorganic P	335 μg./g.	53,300 counts/min./g.	344 μg./g.	15,930 counts/min./g.
Acid-soluble organic P	1040 μg./g.	42,600 counts/min./g.	1170 μg./g.	28,150 counts/min./g.
Acid-insoluble P	374 μg./g.	146,500 counts/min./g.	414 μg./g.	14,370 counts/min./g.

Table 2. *Analyses of muscles soaked in phosphate solutions*

			Phosphate fractions (μg. P/g. muscle, and labelled P) assuming radioactive phosphate identical with chemical phosphate							
			Inorganic P		Phosphocreatine		Pyrophosphate		Debris	
Exp.	Soaking-in solution (μg. P/ml.)	Time of soaking (hr.)	μg./g.	% *P	μg./g.	% *P	μg./g.	% *P	μg./g	% *P
72	50	3	287	1·42	600	0·78	157	0·54	600	0·17
73	50	3	284	0·85	715	1·0	209	0·76	715	0·08
56	100	2·2	145	0·65	690	0·42	200	0·30	327	0·14
57	100	2·5	192	0·69	748	0·59	441	0·43	403	0·20
58	100	2·8	192	0·41	500	0·15	405	0·07	385	0·12
59	100	3·0	200	0·54	424	0·11	200	0·10	327	0·20
52	200	2·5	333	1·45	408	0·30	872	0·13	389	0·31
53	200	3·0	316	1·28	245	0·50	687	0·21	350	0·52
54	200	3·3	261	2·0	500	0·40	690	0·16	327	0·76
55	200	3·6	226	2·3	410	0·56	613	0·19	340	0·50
A	440	1·7	348	2·4	174	0·47	365	0·39	335	0·26
B	440	1·7	348	2·2	174	0·49	508	0·21	412	(2·3)
C	3100	16	388	2·25	248	1·05	348	0·97	415	(5·9)
D	3100	16	523	2·08	151	0·66	475	0·71	388	0·4
E	3100	16	536	3·34	152	1·0	434	0·89	381	(1·9)

The bracketed figures are especially high and almost certainly indicate the presence of trace compounds in the soaking medium. The most striking features of the results are: (*a*) the inorganic phosphate is not much increased by exposure to high concentrations of it; (*b*) the phosphocreatine seems to be diminished as a result of treatment with high phosphate concentrations, possibly as a result of the spontaneous mechanical activity usually set up in these media, presumably because of calcium precipitation; (*c*) the degree of exchange or attachment of the labelled phosphate is not increased proportionately to the increase in the concentration of phosphate in the medium.

The effect of variations of the conditions upon the uptake. By using two sartorii taken from one frog it is possible to make a comparison between the uptakes of labelled phosphate from different media, or from the same media at different temperatures. Experiments made in 0·1 M-potassium phosphate and 0·1 M-sodium phosphate failed to show any difference in behaviour, although the muscles are depolarized in the former medium. Comparison of uptake at 0° with that at 18° also failed to show any difference, the only factor which gave a fairly consistent effect was the pH (Table 3), when this was controlled by variation of the proportion of mono- and di-basic phosphates added to the Ringer solution.

Table 3. *Comparison of pair muscles soaking in media of different* pH, *containing* ^{32}P

(The figures are the ratio labelled phosphate per g. of muscle to labelled phosphate per ml. solution found after washing the muscles to constant activity in an inactive solution.)

Concentration of labelled P in soaking-in solution (μg./ml.)	Muscle 1		Muscle 2	
	pH	Ratio	pH	Ratio
50	7·45	0·11	6·5	0·10
100	7·55	0·031	6·5	0·031
100	7·55	0·078	6·5	0·051
200	7·60	0·065	6·5	0·035
200	7·60	0·13	6·5	0·068
400	7·75	0·043	6·4	0·010
400	7·75	0·085	6·4	0·077
1000	7·80	0·04	6·1	0·009

Experiments with labelled hexosephosphates. Some runs were made using labelled glucose-1- and glucose-6-phosphates, the phosphorus being labelled with ^{32}P. The same general procedure was followed. As the specific activities of the compounds were low a precise measure of the uptake of labelled phosphorus from them was not obtained, but the fact that it was small was of significance. A mixture of isotonic hexosephosphate (1 vol.) with Ringer solution (4 vol.) was used to soak the muscles, and the loss of the radioactivity to ordinary Ringer solution was then observed.

The results given by glucose-1-phosphate indicated an extracellular space of 0·2 by volume, and a diffusion constant for the glucose phosphate not very different from that of inorganic phosphate. The uptake by the cells was of the same order as that from inorganic phosphate, and the soaking-out curve resembled Fig. 2*a*, that is there was no fraction interfering with the extracellular part.

Glucose-6-phosphate, which was kindly prepared by Mr J. H. Ottaway of the Biochemistry Department, also furnished a similar value for the extracellular space and diffusion constant, but it differed in that a part of the labelled phosphate left the muscle with a time constant of about 2 hr. On account of the low activity it is not possible to say whether there was any residual fraction. The result suggests that glucose-6-phosphate may be adsorbed on the cells in a way similar to the readily lost substance present when the curves like Fig. 3 are obtained.

DISCUSSION

The radioautographs indicate that the major part of the labelled phosphate taken up by muscle either *in vivo*, or *in vitro*, is present on the cell surface. It must be kept in mind that the part of the phosphate which is involved in the uptake from the solution is only about 1 % of the total phosphate of the muscle. It is misleading to express the results of phosphate uptake in terms of permeability. Much of the labelled phosphate cannot be removed by exposure to Ringer solution and when the muscle is subjected to the usual analytical procedure the labelled phosphorus appears in the various fractions, that is to say, the surface complex must be capable of yielding a variety of products when treated with trichloroacetic acid. A part of the labelled phosphate remains attached to the debris after extraction, and it was shown by ether-ethanol extraction that it was not present as phospholipin, so it must be held on to the protein or combined as nucleic acid. Marschak & Calvet (1949) noted that as much as a half of the radioactive phosphorus taken up by their rabbit-liver slices remained attached to the acid-insoluble debris, so it appears to be a general property of proteins to retain a proportion of phosphate even after acid extraction. The presence of trace compounds in the ^{32}P solution may account at least in part for anomalies of this sort. Kamen & Spiegelman (1948) have discussed at some length the variation in the degree of extraction with extractant, and the specific activity obtained by serial extraction of phosphate derived from yeast cells previously treated with radioactive media. The difference noted by Govaerts (1948) between the phosphate of the blood stream and the injected labelled phosphate could be explained either by the blood phosphate

being in a combined form (e.g. as a stabilized calcium phosphate sol) or by the radioactive phosphate containing traces of other phosphorus compounds. Another explanation has recently been given by Handler & Cohn (1951).

The bulk of the inorganic phosphate derived from the muscle cells is usually considered to be an artifact formed from some labile compounds during the extraction (Kamen & Spiegelman, 1948; Baldwin, 1947). Our results, with the radioautographs, indicate that a proportion of all the fractions is derived from a layer near the surface, and it is unlikely that the various compounds are present there in an entirely free state; it is more probable that a complex phosphoprotein is their source, and that this complex is only partly broken down by trichloroacetic acid, or other denaturants.

The fact that more phosphate is adsorbed from less acid media than from more acid media (Table 3) agrees with the common experience that phosphate is more adsorbed on to any surface such as glass, from such solutions. The absence of a detectable temperature effect is surprising, for a chemical reaction evidently proceeds leading to the formation of a complex capable of furnishing organic phosphocompounds on treatment with a denaturant. Since the autographs indicate that the major part of the labelled phosphate is situated on the surface of the muscles, earlier work on phosphate uptake has to be interpreted in this light, rather than on the assumption found that equilibration with the internal components of the cell has taken place.

Our results show that more attention must be paid to the conversion of all the ^{32}P of radioactive phosphate samples into orthophosphate before their application in metabolic studies. It is to be emphasized that the complete adsorption by a small muscle of all the trace compound present in a considerable volume of surrounding fluid can give rise to an enormous error if the radioactivity is used as a measure of the orthophosphate present. Suppose a solution with 100 μg. phosphate P, and 1 μc./ml. be used, and that 0·01 μc./ml. be associated with a trace compound. Then the complete adsorption by a muscle of the trace compound present in, say, 100 ml. solution, a volume often employed in these experiments, will give the specimen an activity corresponding to an apparent uptake of 100 μg. phosphate.

SUMMARY

1. The chemical nature of a part of the radioactive phosphate commonly used in tracer experiments is called into question. It appears that evaporation to dryness with acid reduces interference by strongly adsorbable trace compounds.

2. Experiments were made to measure the uptake by frog sartorii of phosphate from solution, and radio-autographs were prepared of specimens which had been washed free from adhering solution. The latter showed that the radioactivity was confined to a layer near the surface of the fibres.

3. The additional activity introduced when trace compounds were in the solution appeared in the trichloroacetic acid-insoluble fraction when analysis was attempted.

4. When muscles prepared by soaking in ^{32}P solution were transferred to a non-radioactive solution, a part of the activity was rapidly lost, this is attributed to extracellular ^{32}P. The behaviour of the residual radioactivity was variable; in certain cases it was all retained by the muscle whereas in others some was lost over a period of 1–2 hr., which made the estimation of the extracellular space difficult and subject to error.

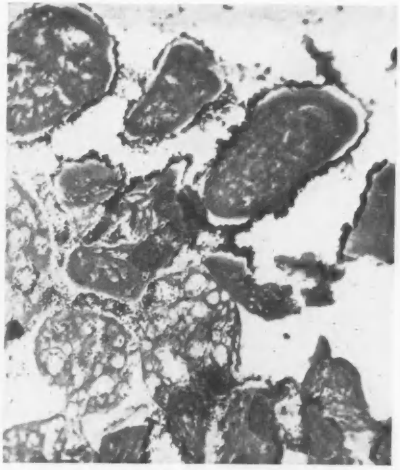
5. Analysis of the specimens after soaking in a radioactive phosphate medium showed the presence of isotopically distinguished phosphate in the organic compounds as well as in inorganic and trichloroacetic acid insoluble fractions.

6. The fact that the radioactive phosphorus, shown by the autographs to be on or near the cell surfaces, appears in the organic P fractions is taken as evidence for the phosphorus being held as a complex compound on, and in, the protein.

We should like to thank Mr G. P. Burn for carrying out the analyses. The radioactive phosphate was obtained from the Atomic Energy Research Establishment. One of us (E. J. H.) is in receipt of an expenses grant from the Royal Society.

REFERENCES

Baldwin, E. (1947). *Dynamic Aspects of Biochemistry.* Cambridge University Press.
Eggleton, M. G. (1933). *J. Physiol.* **79**, 31.
Furchgott, R. F. & Shorr, E. (1943). *J. biol. Chem.* **151**, 65.
Govaerts, J. (1948). *Arch. int. Pharmacodyn.* **75**, 261.
Hahn, P. F., Hevesy, G. & Rebbe, O. (1939). *Biochem. J.* **33**, 1549.
Handler, P. & Cohn, D. V. (1951). *Amer. J. Physiol.* **164**, 646.
Harris, E. J. & Burn, G. P. (1949). *Trans. Faraday Soc.* **45**, 508.
Hevesy, G. & Rebbe, O. (1940). *Acta physiol. scand.* **1**, 171.
Kalckar, H. M., Dehlinger, J. & Mehler, A. (1944). *J. biol. Chem.* **154**, 275.
Kamen, M. D. & Speigelman, S. (1948). *Cold Spr. Harb. Sym. quant. Biol.* **13**, 151.
Krogh, A. (1946). *Proc. roy. Soc.* B, **133**, 140.
Manery, J. F. & Bale, W. F. (1941). *Amer. J. Physiol.* **132**, 215.
Marschak, A. & Calvet, F. (1949). *J. cell. comp. Physiol.* **34**, 451.
Rothstein, A. & Meier, R. (1948). *J. cell. comp. Physiol.* **32**, 77.
Sacks, J. (1948). *Cold Spr. Harb. Sym. quant. Biol.* **13**, 180.
Wood, H. G. (1948). In *Isotopes in Biology and Medicine.* Madison: University of Wisconsin Press.



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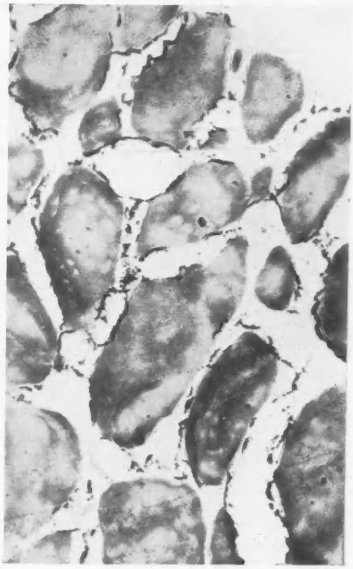

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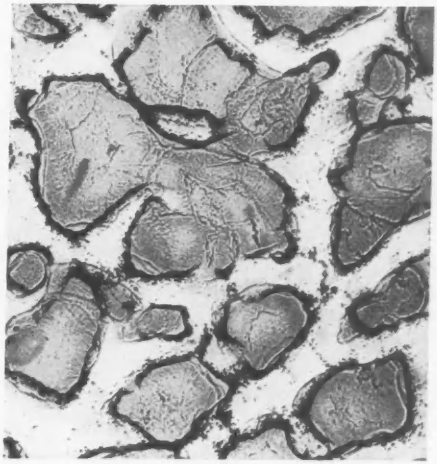

D

G. CAUSEY AND E. J. HARRIS—THE UPTAKE AND LOSS OF PHOSPHATE BY FROG MUSCLE

EXPLANATION OF PLATE 1

A. Frog muscle prepared by injection and cut frozen (×175.)

B. Frog muscle prepared by injection and cut frozen. (×175.)

C. Longitudinal section of rat muscle prepared by injection, and cut after fixation. (×280.)

D. Frog muscle prepared by soaking and cut after fixation. (×280.)

The Action of Urea on the Disulphide Groups of Oxidized Glutathione and Cystine

By R. CECIL
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In a previous paper (Cecil, 1950) it was suggested that the differences in reactivity between oxidized and reduced glutathione (G_2S_2 and GSH) on the one hand, and cystine and cysteine on the other, could be explained by hydrogen bonding between the sulphur and the glutamyl-NH_3^+ of the glutathione. If this explanation is correct then any substance which competes with the sulphur, by itself forming hydrogen bonds with the glutamyl-NH_3^+, should tend to reduce this difference in reactivity. Crystalline urea forms intermolecular N—H—O bonds (Wyckoff & Corey, 1934; Pauling, 1940) and it is likely that this also obtains to some extent in solution, particularly at high concentrations. It is therefore to be expected that urea will form hydrogen bonds with the glutamyl-NH_3^+ of G_2S_2 and so tend to make it behave like cystine.

It was shown by Cecil (1950) that the hydrolytic fission reaction of disulphides with an excess of silver nitrate can be used as a measure of comparative disulphide reactivity. The reaction, which may be represented by the following equations:

$$2RSSR + 2H_2O \rightleftharpoons 2RSH + 2RSOH, \quad (1)$$

$$2RSOH \rightleftharpoons RSH + RSO_2H, \quad (2)$$

$$3RSH + 3Ag^+ \rightarrow 3RSAg + 3H^+, \quad (3)$$

was followed by estimating the remaining free silver ion by potentiometric titration against potassium bromide.

Three different types of reaction kinetics were described and arbitrarily classified as types I, II and III. The type I reactions are unimolecular with respect to disulphide, (1) being the limiting process. Type II reactions are not unimolecular, suggesting that the dismutation (2) is the limiting process. Type III reactions occur at low pH and differ from I and II in that they do not go to completion. Under the conditions chosen for this work G_2S_2 gives a type I reaction, and cystine a much slower type II reaction. This paper describes the effect of urea on these reactions.

EXPERIMENTAL

Reaction conditions

The apparatus and methods used were described in the previous paper (Cecil, 1950). The reactions were carried out in the dark at 37° in the range pH 7·0–7·6. α-Picoline, 0·02 M, was used as the buffer, the acid component being either cystine or G_2S_2. The concentrations of G_2S_2 and cystine were approximately 2×10^{-4} M, and that of the $AgNO_3$ 10^{-3} M. When high urea concentrations (4–9 M) were used the reactions were carried out in 10 ml. Pyrex standard flasks. The urea was added as solid and the other reagents in solution. At lower urea concentrations Pyrex boiling tubes were used as previously.

Purification of reagents

Urea. This was twice recrystallized from hot ethanol. The ethanol used was first refluxed over Zn and NaOH, to remove chloride and peroxides, and redistilled. The halide content was always less than 1 part of Cl^- in 10^6, measured as described by Cecil (1950).

α-Picoline. The sample used in the previous work gave no reaction with $AgNO_3$. In the presence of urea, however, a slow reaction took place involving an appreciable Ag uptake. This was shown to be due to an impurity in the picoline, which was removed by refluxing over Ag_2O for 2 hr. and redistilling. The fraction boiling at 128–130° was used.

G_2S_2 and cystine. The samples described in the previous paper (Cecil, 1950) were used.

Stability of solutions

Dilute standard solutions are always liable to error because of adsorption on glass. In sunny weather particularly, $AgNO_3$ solutions were found to lose 3–4 % of their Ag content during the day, which reappeared in solution overnight. The use of coloured bottles reduced, but did not eliminate, this effect. As a precaution the G_2S_2 and cystine solutions were

checked each day, and the $AgNO_3$ immediately before each experiment, using the methods described by Cecil (1950). Tests were also made with the Pyrex vessels used for the reactions. Under the conditions used, namely 37° in the dark, no adsorption was found.

Measurement of pH

pH measurements were made with a glass electrode calibrated with 0·05 M-potassium acid phthalate, taken as pH 4·00 at 20° (MacInnes, Belcher & Shedlovsky, 1938), and 0·05 M-$Na_2B_4O_7.10H_2O$ taken as pH 9·18 at 20° (Lauchlan, 1944). It was found that at the high urea concentrations the pH of the reaction mixtures rose to 7·6 as against 7·0 in the absence of urea. This effect was confirmed with a hydrogen electrode. Urea has no buffering power in this pH range and so this difference must have been due to the effect of urea on the dissociation constants of the ionizing groups present. Although the addition of acid would correct the pH readings it would not restore the original dissociation constants. Since there was therefore no way of making the reaction conditions identical in the presence and absence of urea, it was decided to maintain the same buffer concentrations throughout.

Previous work (Cecil, 1950) had shown that the hydrolytic fission of disulphides is favoured by increasing pH, and so it is possible that the effect of urea in slowing this reaction is greater than appears, but it cannot be less.

RESULTS AND DISCUSSION

The results are expressed graphically on the basis of the kinetic treatment given in the previous paper (Cecil, 1950). Log $(c-x)$ is plotted against time, where c = initial concentration of disulphide in equivalents, and x = reacted concentration in equivalents. Type I (unimolecular) reactions will therefore give linear, and types II and III curved plots.

G_2S_2 normally gives a type I reaction at pH 7. The effect of 1 M-urea is only just detectable, but as the concentration is raised from 4 to 9 M the reaction is progressively reduced in rate and becomes type II (Fig. 1). Previous work shows that, apart from interaction between the urea and G_2S_2, this effect could be caused either by lowered pH, already discussed, or decreased silver ion concentration.

If urea binds silver ion sufficiently to interfere with the latter's reaction with GSH, then urea should also interfere with the silver ion reaction with bromide ion, since silver electrode potential measurements show that GSH has a higher affinity for silver ion than has bromide ion. Accordingly, silver nitrate was titrated with potassium bromide in the presence of 10 M-urea at a pH of approximately 7·5. The titre, determined by the potentiometric method, was 3 % low, which is little more than the error (1–2 %) due to the use of the silver electrode at this pH. At low pH values (2–3) this error disappears completely. It is, therefore, considered unlikely that decreased silver ion concentrations are responsible for the effects observed.

Experiments were tried with G_2S_2 in 6 M-urea in which the urea, G_2S_2, and picoline were incubated at 37° for periods of up to 3 hr. before adding the silver nitrate. The effect was the same as when the silver nitrate was added simultaneously with the other reagents, showing that the action of urea on G_2S_2 is rapid. The reverse effect, namely the return of G_2S_2 to normal behaviour, is also rapid. Solid urea (0·6 g.) was added to 2 ml. of $9{\cdot}6 \times 10^{-4}$ M-G_2S_2 solution, giving a urea concentration of approximately 4 M, and this solution was incubated for 1 hr. at 37°. The total volume was then made up to 10 ml. by adding α-picoline and silver nitrate solutions and water, so that the final concentrations of all reagents was the same as in the previous experiment in 1 M-urea. The same reaction rate was obtained.

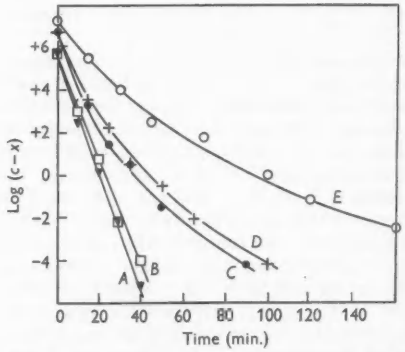

Fig. 1. Comparative kinetics of the G_2S_2-$AgNO_3$ reaction at pH 7·0–7·6 in the presence of varying concentrations of urea. *A*, ▼, control reaction without urea; *B*, □, reaction in 1 M-urea; *C*, ●, reaction in 4 M-urea; *D*, +, reaction in 6 M-urea; *E*, ○, reaction in 9 M-urea.

The action of urea on the cystine-silver nitrate reaction was also investigated. Previous work (Cecil, 1950) had shown that cystine is normally more sensitive in this reaction to added reagents than is G_2S_2. Thus acetate ion catalyses the cystine reaction quite markedly, but the G_2S_2 one very little. Barbitone slows the G_2S_2 reaction, but completely stops that of cystine. The impurity in the α-picoline, mentioned earlier, has no effect on the G_2S_2 reaction, but has considerable effect on that of cystine. This last effect was given as a correction in proof in the previous paper.

Fig. 2 shows the correct curve for the cystine-silver nitrate reaction in α-picoline at pH 7·0. Addition of 10^{-3} M-acetate swamps the effect of the impurity, the same increased rate being obtained whether the pure or impure picoline is used.

Unlike acetate and barbitone, urea has much less effect on cystine than G_2S_2. The results for cystine

(Fig. 2) show a slight reduction in rate in the presence of 1 M-urea, but no further reduction when the urea concentration is raised to 6 M. In both cases the reaction remains type II. No effect was observed with 0·2 M-urea.

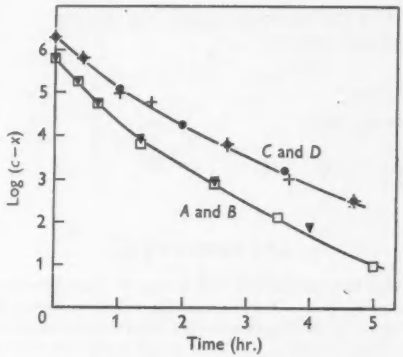

Fig. 2. Comparative kinetics of the cystine-$AgNO_3$ reaction at pH 7·0–7·6 in the presence of varying concentrations of urea. *A*, ▼, control reaction without urea; *B*, □, reaction in 0·2 M-urea; *C*, ●, reaction in 1 M-urea; *D*, +, reaction in 6 M-urea.

While these various results cannot be said to prove the hypothesis put forward earlier, they are in agreement with it. Thus, if the action of urea on G_2S_2 is to break N—H—S bonds between the sulphur and the glutamyl-NH_3^+ by competitive action, one would expect G_2S_2 to behave increasingly like cystine as the urea concentration is raised. This does in fact happen. The reaction slows progressively and changes from type I to type II, though, even when the urea concentration is 9 M, G_2S_2 reacts considerably more rapidly than cystine. The latter suggests that the glutamyl-NH_3^+ forms more stable bonds with the sulphur than with urea. This is in accord with the assumption that the sulphur bonds are formed by chelation, whereas those with urea are intermolecular. It is thought possible that this effect of urea on glutathione may be related to its well known effect on the sulphur groups of certain proteins first reported by Ramsden (1940).

The effect on cystine is much smaller, and is maximal with 1 M-urea. This effect differs from the inhibitory action of barbitone, or the catalytic action of GSH and G_2S_2 (Cecil, 1950). These three substances all form moderately stable complexes with cystine and in consequence they are effective at concentrations comparable with that of cystine, whereas urea shows no appreciable effect at 0·2 M. A possible explanation is that cystine exhibits some intermolecular N—H—S bonding in solution, insufficient to affect markedly its chemical reactivity, and which is easily interfered with by urea.

SUMMARY

1. The kinetics of the reaction between oxidized glutathione and silver nitrate at pH 7·0–7·6 were studied in the presence of varying concentrations of urea. Urea concentrations of 4 M and above progressively reduced the reaction rate and changed the kinetics towards those characteristic of the cystine-silver nitrate reaction.

2. The rate of the cystine-silver nitrate reaction was slightly reduced by 1 M-urea, but no further effect was produced by raising the urea concentration to 6 M. The character of the kinetics was unchanged.

3. It is suggested that the urea acts by breaking N—H—S bonds between the sulphur and the glutamyl-NH_3^+ of the oxidized glutathione, thus making it behave like cystine. The existence of this N—H—S bond was discussed in a previous paper. The smaller effect of urea on cystine suggests that this substance normally exhibits some intermolecular N—H—S bonding which hardly influences its chemical behaviour.

REFERENCES

Cecil, R. (1950). *Biochem. J.* **47**, 572.
Lauchlan, A. D. E. (1944). *Nature, Lond.*, **154**, 577.
MacInnes, D. A., Belcher, D. & Shedlovsky, T. (1938). *J. Amer. chem. Soc.* **60**, 1099.
Pauling, L. (1940). *Nature of the Chemical Bond*, 2nd ed. p. 315. Cornell: University Press.
Ramsden, W. (1930). *Chem. & Industr.* **8**, 851.
Wyckoff, R. W. G. & Corey, R. B. (1934). *Z. Krystallogr.* **89**, 462.

Studies on the Biochemistry of Zinc

2. THE EFFECT OF INTAKE OF ZINC ON THE METABOLISM OF RATS MAINTAINED ON A STOCK DIET

By V. SADASIVAN*
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(*Received* 22 *November* 1950)

In a previous paper (Sadasivan, 1951), the effects of zinc on rats maintained on a stock diet and a high-fat, low-protein diet were described. It was shown that zinc in excess adversely affected the growth rate of the animals (cf. Smith & Larson, 1946) and that both the weight of the liver and its fat content decreased appreciably. Analysis of the femurs of experimental animals showed that zinc also interfered with the development and mineralization of bones.

The present experiments were carried out on rats to find out what metabolic changes were caused by the addition of excess zinc to a stock diet. In assessing the changes in metabolism, attention was paid mainly to the assimilation and excretion of nitrogen, phosphorus and sulphur and the urinary excretion of uric acid and total creatinine (creatine and creatinine). The changes in nitrogen and sulphur were followed because it has been fairly clearly shown that the sulphur amino-acids play a decisive role in the transport of fat from liver and also because sulphur amino-acids have a considerable influence on nitrogen balance in the animal. The reason for a study of phosphorus metabolism will be obvious from the report in the previous paper that zinc affects bone development. Moreover, the studies on the lipotropic activity of choline and methionine by Aylward, Channon & Wilkinson (1935) and Perlman & Chaikoff (1939*a*, *b*, 1940) suggest that phosphorus transformations play an important part in fat metabolism.

It has been pointed out by Holmberg (1939) and Davidson (1942) that uricase may be a zinc enzyme. Wachtel, Hove, Elvehjem & Hart (1941), however, could not demonstrate any effect of zinc deficiency on the uric acid concentration of blood. In the present work, it was thought desirable to find out if, with comparatively large concentrations of zinc in the diet, the formation of uric acid was influenced. Uric acid is easily eliminated in the urine, and urinary excretion was therefore followed; the elimination of total creatinine was also determined.

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EXPERIMENTAL

In detail the experiments were similar to those described in the previous paper (Sadasivan, 1951) with the exception that the rats were placed in metabolism cages and urine and faeces were collected daily. For each 3-day period, for each rat, urine samples and faeces samples were pooled. Twelve rats from two litters were divided into three groups of four with an equal distribution of sexes and litter mates. Records of weight and of food intake were kept for each 3-day period for each rat.

Uric acid was determined by the colorimetric method of Folin (1933) using urea-cyanide mixture to prevent the formation of precipitates. Total creatinine was determined by the Kjeldahl method in samples of the urine, faeces or diet. Phosphorus and sulphur were determined by mixing samples of the urine, faeces or diet with adequate quantities of pure $Mg(NO_3)_2$, evaporating the mixture to dryness on a sand bath and ashing the material in a muffle furnace at low red heat. The ash was then extracted with dilute HCl, filtered and made up to a standard volume from which samples were taken for colorimetric determination of P by the method of Fiske & Subbarow (1925) while sulphur was determined gravimetrically as $BaSO_4$.

RESULTS

The growth rate of the rats and their average food intake for each 3-day period are shown in Fig. 1, from which it is seen that while growth rate is progressively decreased with increase in level of supplement, food intake is diminished only at the higher level of supplement of zinc.

Nitrogen metabolism. The average results of urinary and faecal excretion of nitrogen and nitrogen retention for each 3-day period are shown in Figs. 2–4. It will be seen from these results that zinc oxide at the 0·5 % level did not alter appreciably the urinary excretion or retention of nitrogen, but there was an increase in faecal nitrogen. On the other hand, supplements at the 1·0 % level of zinc oxide caused increased excretion of nitrogen both in urine and faeces, and a lower nitrogen retention than in the control animals.

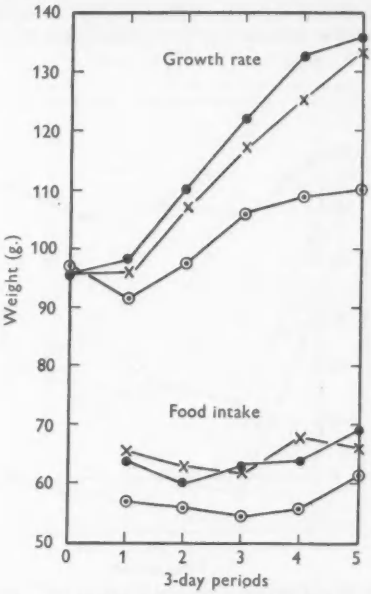

Fig. 1. Average food intake and growth rate of rats receiving zinc as supplement to a stock diet. ●—●, stock diet alone; ×—×, stock diet supplemented with zinc oxide (0·5%); ⊙—⊙, stock diet supplemented with zinc oxide (1·0%).

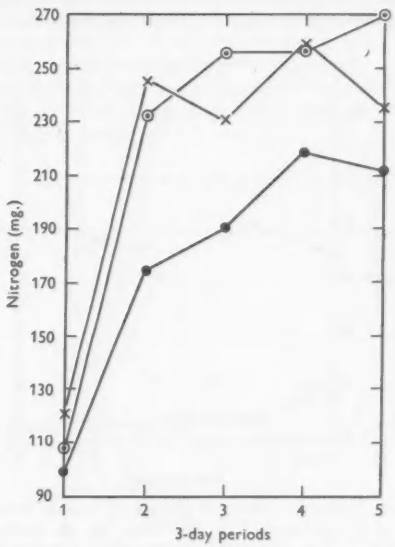

Fig. 3. Faecal excretion of nitrogen in rats fed on a zinc-supplemented diet. ●—●, stock diet alone; ×—×, stock diet supplemented with zinc oxide (0·5%); ⊙—⊙, stock diet supplemented with zinc oxide (1·0%).

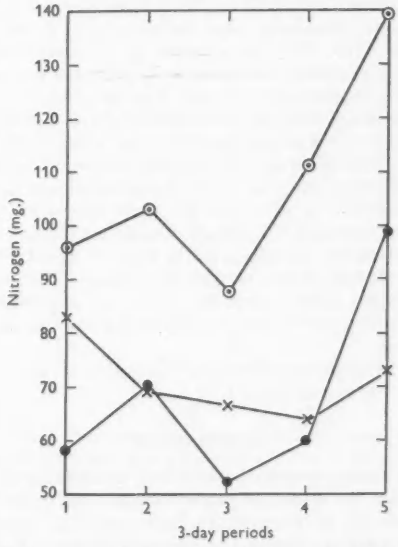

Fig. 2. Average urinary excretion of nitrogen in rats fed on a zinc-supplemented diet. ●—●, stock diet alone; ×—×, stock diet supplemented with zinc oxide (0·5%); ⊙—⊙, stock diet supplemented with zinc oxide (1·0%).

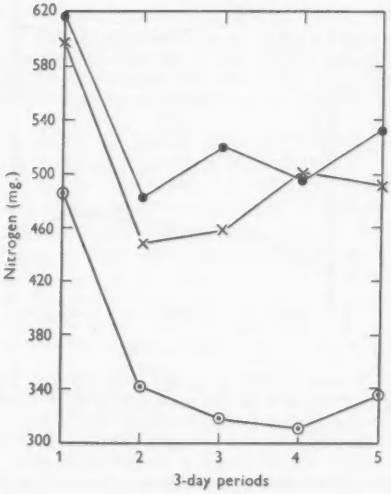

Fig. 4. Nitrogen retention in rats fed on a zinc-supplemented diet. ●—●, stock diet alone; ×—×, stock diet supplemented with zinc oxide (0·5%); ⊙—⊙, stock diet supplemented with zinc oxide (1·0%).

Phosphorus metabolism. The elimination and retention of phosphorus was followed and the results are presented in Figs. 5–7. Zinc supplements bring about a decrease in the urinary excretion of phosphorus with simultaneous increase in the faecal phosphorus. This change was seen when zinc oxide was fed at the 0·5 % level and urinary elimination did not decrease further with increasing level of supplement; faecal excretion did, however, show an increase. The retention of phosphorus also decreased with the higher level of supplement.

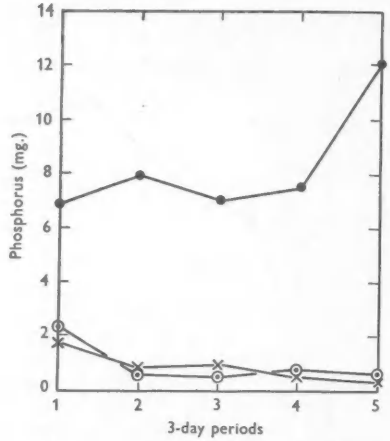

Fig. 5. Urinary excretion of phosphorus in rats receiving zinc as supplement to a stock diet. ●—●, stock diet alone; ×—×, stock diet supplemented with zinc oxide (0·5 %); ⊙—⊙, stock diet supplemented with zinc oxide (1·0 %).

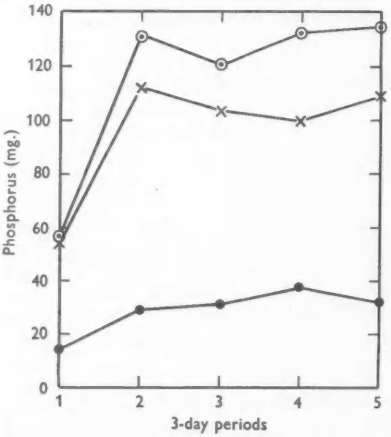

Fig. 6. Faecal excretion of phosphorus in rats on a zinc-supplemented diet. ●—●, stock diet alone; ×—×, stock diet supplemented with zinc oxide (0·5 %); ⊙—⊙, stock diet supplemented with zinc oxide (1·0 %).

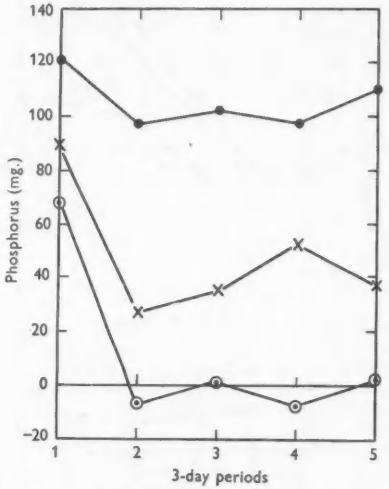

Fig. 7. Phosphorus retention in rats fed a zinc-supplemented diet. ●—●, stock diet alone; ×—×, stock diet supplemented with zinc oxide (0·5 %); ⊙—⊙, stock diet supplemented with zinc oxide (1·0 %).

Sulphur metabolism. The quantities of total sulphur eliminated and retained are shown in Figs. 8–10. The metabolism of sulphur follows almost the same course as that of phosphorus. Thus, while supplements of zinc lowered the urinary excretion of sulphur, there was an increase in faecal excretion and decrease in retention. Urinary elimination of sulphur did not show any marked difference between the lower and higher supplements of zinc.

Excretion of uric acid and total creatinine. The observations on the urinary elimination of these two constituents are presented in Figs. 11 and 12. The elimination of uric acid shows a general but small increase with increasing level of supplement. Excretion of total creatinine also followed the same course.

The results for the entire experimental period are summarized in Table 1.

DISCUSSION

The results presented show that zinc affects metabolism to a considerable extent. The increased excretion of nitrogen in both urine and faeces, resulting in lowered retention in the animals, suggests a general wastage of tissue proteins. Nitrogen excretion in both urine and faeces is higher with the higher level of supplementary zinc. The

increased excretion of uric acid and total creatinine, although slight, also points to an enhanced catabolism.

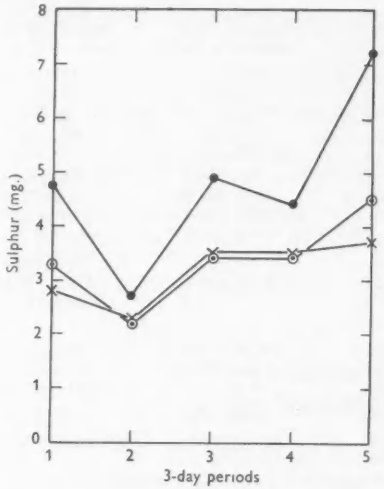

Fig. 8. Urinary excretion of sulphur in rats receiving stock diet supplemented with zinc. ●—●, stock diet alone; ×—×, stock diet supplemented with zinc oxide (0·5%); ⊙—⊙, stock diet supplemented with zinc oxide (1·0%).

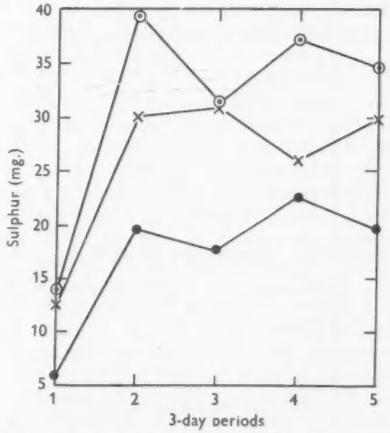

Fig. 9. Faecal excretion of sulphur in rats fed a zinc-supplemented diet. ●—●, stock diet alone; ×—×, stock diet supplemented with zinc oxide (0·5%); ⊙—⊙, stock diet supplemented with zinc oxide (1·0%).

Zinc supplements bring about a lowered elimination of phosphorus and sulphur in the urine irrespective of the level of zinc supplement, but faecal excretion is increased to such an extent that there is lowered retention. This is probably one of the causes of poor development of bones (Sadasivan, 1951). It is not clear, however, whether zinc exercises any direct action on the bones by mobilizing calcium and phosphorus from them. From the fact that urinary phosphorus is decreased in zinc-supplemented rats, it has to be inferred that the minerals of the bones are not directly mobilized.

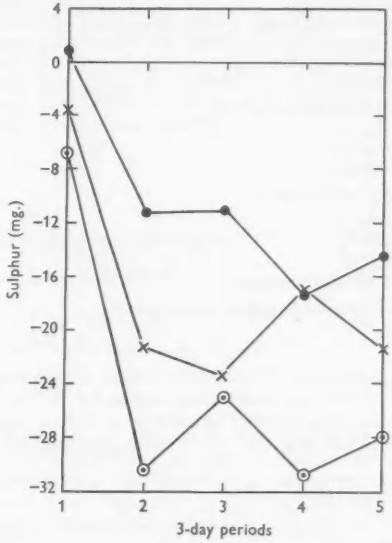

Fig. 10. Sulphur retention in rats receiving stock diet with supplements of zinc. ●—●, stock diet alone; ×—×, stock diet supplemented with zinc oxide (0·5%); ⊙—⊙, stock diet supplemented with zinc oxide (1·0%).

The results on faecal excretion of phosphorus suggest that zinc prevents assimilation of phosphorus either by interfering with its transport through the walls of the intestines, or by precipitating it as insoluble phosphates of zinc in a manner analogous to that demonstrated by Kay & Skill (1934) in 'beryllium rickets'. The likelihood of phosphorus compounds being precipitated by zinc in the intestines appears to be small. In our stock diet, phosphorus is mainly in organic combination; there is, however, the possibility that organic phosphorus compounds in the diet are hydrolysed by the intestinal phosphatases. Determinations of phosphatase activity in the tissues and bones of rats have shown that the enzyme in the intestines is appreciably decreased in zinc-fed rats. These results will be presented in a later paper.

The increased faecal excretion of phosphorus in zinc-supplemented animals affords an explanation for the poor development of bones reported in the

Table 1. *Influence of zinc on the metabolism of rats fed on a stock diet*

(The results are for the whole period of the experiment and the average for four rats in each group.)

	Stock diet alone	Stock diet supplemented with 0·5% ZnO	Stock diet supplemented with 1·0% ZnO
Increase in weight of rats (g.)	40·5 (±3·9)	38·0 (±3·6)	13·0 (±4·7)
Daily food intake (g.)	21·4 (±0·59)	21·7 (±0·71)	19·0 (±0·54)
Nitrogen balance (mg.)			
Urinary N	339±84·5	355±56·5	536±140·3
Faecal N	840±48·6	1091±91·9	1122±72·4
Retention	2647±146	2486±90·3	1791±159·2
Phosphorus balance (mg.)			
Urinary P	41·4±9·7	4·3±0·24	4·6±0·63
Faecal P	143·8±6·2	477·8±20·4	573·2±30·2
Retention	526·4±18·5	239·5±23·8	54·8±10·1
Sulphur balance (mg.)			
Urinary S	23·9±5·34	15·8±1·06	16·8±2·65
Faecal S	86·9±3·4	129·3±3·95	155·5±7·3
Retention	−53·1±4·63	−86·5±3·8	−121·0±7·23
Uric acid excretion (mg.)	3·89±0·82	4·29±0·62	5·45±1·0
Total creatinine (creatine + creatinine) (mg.)	12·61±3·2	14·40±2·2	20·61±4·3

previous paper. The lowered intake of phosphorus compounds may also be responsible for the lowered fat content of livers of animals after feeding a high-fat, low-protein diet. The results presented in this paper show that zinc supplements retard the assimilation of food constituents from the intestines in almost the same way as zinc deficiency (cf. Hove, Elvehjem & Hart, 1937).

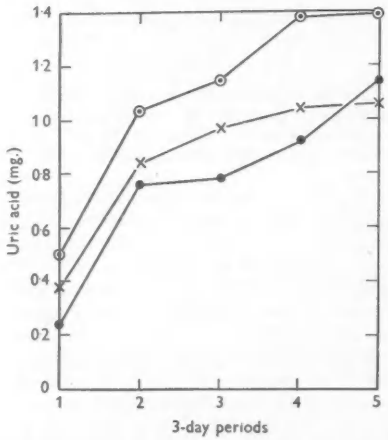

Fig. 11. Urinary excretion of uric acid in rats fed a zinc-supplemented diet. ●—●, stock diet alone; ×—×, stock diet supplemented with zinc oxide (0·5%); ⊙—⊙, stock diet supplemented with zinc oxide (1·0%).

SUMMARY

1. Experiments have been made on rats on a stock diet supplemented with 0·5 and 1% of zinc oxide.

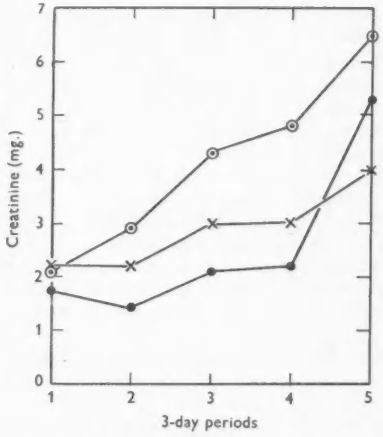

Fig. 12. Urinary excretion of total creatinine in rats on a zinc-supplemented diet. ●—●, stock diet alone; ×—×, stock diet supplemented with zinc oxide (0·5%); ⊙—⊙, stock diet supplemented with zinc oxide (1·0%).

2. Urinary and faecal nitrogen excretion increased as a result of the zinc supplement.

3. Urinary excretion of phosphorus and sulphur was lowered, but faecal excretion was increased.

4. Urinary excretion of uric acid and of creatinine increased on a zinc-containing diet.

5. These results are discussed in relation to the systemic effects previously noted by the author as characteristic of zinc poisoning.

I am grateful to Major-General S. S. Sokhey, Director, and Dr K. Ganapathi, Assistant Director, for their interest in the work.

REFERENCES

Aylward, F. X., Channon, H. J. & Wilkinson, H. (1935). *Biochem. J.* **29**, 169.
Davidson, J. N. (1942). *Biochem. J.* **36**, 252.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Folin, O. (1933). *J. biol. Chem.* **101**, 111.
Holmberg, C. G. (1939). *Biochem. J.* **33**, 1901.
Hove, E., Elvehjem, C. A. & Hart, E. B. (1937). *Amer. J. Physiol.* **119**, 768.
Kay, H. D. & Skill, D. I. (1934). *Biochem. J.* **28**, 1222.
Perlman, I. & Chaikoff, I. L. (1939*a*). *J. biol. Chem.* **127**, 211.
Perlman, I. & Chaikoff, I. L. (1939*b*). *J. biol. Chem.* **128**, 735.
Perlman, I. & Chaikoff, I. L. (1940). *J. biol. Chem.* **133**, 651.
Sadasivan, V. (1951). *Biochem. J.* (in the Press).
Smith, S. E. & Larson, E. J. (1946). *J. biol. Chem.* **163**, 29.
Wachtel, L. W., Hove, E., Elvehjem, C. A. & Hart, E. B. (1941). *J. biol. Chem.* **138**, 361.

The Preparation of Soluble Cholinesterases from Mammalian Heart and Brain

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In a previous paper (Ord & Thompson, 1950) evidence was presented in support of the presence of two different types of cholinesterase in certain tissues of the rat and of man. One enzyme hydrolyses benzoylcholine and is highly sensitive to inhibition by di-*iso*propyl fluorophosphonate (DFP), and therefore corresponds in these respects to the so-called 'non-specific' (Nachmansohn & Rothenberg, 1945) or 'pseudo' cholinesterase (Mendel & Rudney, 1943), and another, apparently a 'specific' or 'true' cholinesterase, is relatively insensitive to inhibition by DFP and inactive towards benzoylcholine, although readily hydrolysing acetyl-β-methylcholine ($[Me_3N^+.CH_2.CHMe.OAc]OH^-$).

This earlier work was done with whole homogenates of the various blood-free tissues, and it seemed desirable, therefore, to attempt to obtain soluble, purified preparations in order to characterize more clearly the different types of cholinesterase in these tissues. Since rat heart hydrolysed benzoylcholine very actively, we have chosen this tissue as a source of the pseudo-cholinesterase. The rate of hydrolysis of acetyl-β-methylcholine by rat heart, however, is so low as to render it unsuitable for the purification of the true enzyme, and for this purpose we therefore chose rat brain.

A secondary purpose underlying the work described in this paper was provided by considerations as to the toxicity of cholinesterase inhibitors. On account of the selective action of certain inhibitors on the two types of cholinesterase, and of the very different toxicological actions of different cholinesterase inhibitors in the whole animal, it would appear that at least two distinct lines of approach present themselves regarding the mechanism of poisoning: (1) an *in vitro* study of the action of these toxic compounds on the types of cholinesterase present in affected tissues, and (2) a study of these compounds in the whole animal, with a view to determining whether there exist any differences in the power of penetration of different inhibitors to the cholinesterases in different tissues, i.e. whether there are any 'barriers' of a physico-chemical or more complex biological nature that might influence the reactivity of inhibitors with the cholinesterases situated at certain sites in the body. With regard to the first of these alternatives, it is clearly desirable that the enzyme under study should be in a soluble form, so that difficulties of penetration and the influence of adjacent substances situated in cells or cell particles may be reduced to a minimum.

Much work has been done on the purification and behaviour of the soluble cholinesterase of plasma (Stedman & Stedman, 1935; McMeekin, 1939; Strelitz, 1944; Adams & Whittaker, 1949*a*), but some doubt has justifiably been felt about the relevance of such work to the problem of the particular cholinesterase at or near the nerve endings in the tissues. Similarly, purified preparations of erythrocyte cholinesterase have been

described (Mendel & Rudney, 1943; Adams, 1949), but the only purification of a cholinesterase from an innervated mammalian tissue, of which we are aware, is that reported by Mendel & Mundell (1943). These workers prepared a soluble, highly purified pseudo-cholinesterase from dog pancreas. As their source was a glandular tissue, whose exocrine secretion is rich in enzymes, it seemed important to study also a pseudo-cholinesterase derived from a muscular tissue devoid of secretory activity.

Nachmansohn & Rothenberg (1947) obtained highly purified preparations of the true cholinesterase of the electric organ of *Electrophorus electricus*, but no reports have yet been published of any real purification of the enzyme in mammalian brain. Indeed, until the work of Little (1948), it was generally thought that the cholinesterase of brain was firmly associated with the insoluble cell particles of homogenates of this tissue.

EXPERIMENTAL

White rats of either sex were used. They were killed by decapitation, and homogenates of brain or of heart ventricle were made as previously described (Ord & Thompson, 1950).

Estimation of esterase activity. Ammon's (1933) adaptation of the Warburg manometric technique was used. All measurements were made at 38° and at pH 7·4, and were corrected for non-enzymic hydrolysis of the substrate. Esterase activity is expressed as μl. CO_2/g. wet wt. of tissue/hr. for the unpurified preparations, and as μl. CO_2/mg. trichloroacetic acid-precipitable N/hr. for the purified preparations, unless otherwise stated.

Estimation of nitrogen. After precipitation by the addition of an equal vol. of 10% trichloroacetic acid, protein N was estimated by the micro-Kjeldahl technique, using the distillation apparatus described by Markham (1942).

Substrates. (1) Acetylcholine chloride (ACh) (British Drug Houses Ltd.); (2) acetyl-β-methylcholine chloride (MCh) (Savory & Moore, Ltd.); (3) benzoylcholine chloride (BCh), prepared by Dr A. H. Ford-Moore, Experimental Station, Porton; (4) propionylcholine perchlorate (PrCh), and (5) butyrylcholine chloride (BuCh), both given to us by Dr V. P. Whittaker, Department of Biochemistry, Oxford; (6) tributyrin (TB) (British Drug Houses Ltd.).

Each substrate, with the exception of TB, was dissolved in 0·025 M-$NaHCO_3$ immediately before use. The TB was pipetted directly into the side arm of the Warburg flasks, and was covered with a small volume of 0·025 M-$NaHCO_3$. The final concentrations used throughout were 0·015 M for ACh and BCh, and 0·03 M for MCh, PrCh, BuCh and TB, these concentrations giving a steady rate of CO_2 evolution over the times studied.

Inhibitors (1) Di-*iso*propyl fluorophosphonate (DFP), kindly provided by the Experimental Station, Porton; (2) eserine sulphate (British Drug Houses Ltd.). Both inhibitors were dissolved in 0·025 M-$NaHCO_3$ before use.

Other reagents. The reagents used in the course of the purification were all of A.R. standard (British Drug Houses Ltd., and Hopkins and Williams Ltd.). Cholic acid and sodium deoxycholate, prepared from hog bile, were given to us by Prof. G. A. D. Haslewood. Most of the other surface-active substances were kindly provided by Imperial Chemical Industries Ltd.

RESULTS

Rat-heart cholinesterase

Purification. The starting material was a 10% (w/v) homogenate of rat-heart ventricle in 0·025 M-sodium bicarbonate, the final mixture having a pH of 8·4–8·6 and a cholinesterase activity, with acetylcholine as substrate, of about 3600 μl. carbon dioxide/g. wet wt. of tissue/hr. If such a suspension is centrifuged at 3500–4000 rev./min. for 10 min., the greater part of the activity appears in the centrifuged deposit. Following the observation of McCance (1950) that the cholinesterase of dog colostrum is not destroyed by the enzymes of an acetone-dried preparation of pig pancreas, it was decided to attempt to release the cholinesterase from the bulk of material thrown down on centrifuging by carrying out a brief tryptic digestion of the heart homogenates.

A volume of the homogenate, varying in different experiments from 10 to 100 ml., was therefore incubated at 37° for 1 hr. with 1–2 ml. of an aqueous suspension of trypsin (British Drug Houses Ltd.), to give a final concentration of 50 mg. trypsin/100 ml. homogenate. After incubation the overall cholinesterase activity was unchanged, but 88% of it was now present in the supernatant after centrifuging, as compared with only 39% initially (Table 1). In the concentration employed the preparation of trypsin showed no cholinesterase activity.

The supernatant (S_1), which was still very opaque, was separated from the centrifuged deposit; on

Table 1. *Effect of tryptic digestion* (1 *hr. at* 37°) *on the cholinesterase of rat-heart ventricle*

(Activity as μl. CO_2/1·5 ml. homogenate/15 min.)

Whole homogenate			Supernatant from centrifuged homogenate			
A Initially	*B* After tryptic digestion	*B* as % of *A*	*C* Initially	*D* After tryptic digestion	*C* as % of *A*	*D* as % of *A*
178	167	93	72	149	40	84
163	158	97	64	147	39	90
141	157	111	52	128	37	91
	Mean	97			39	88

Table 2. *Stages in purification of rat-ventricle cholinesterase*

(Activity expressed as μl. CO_2/mg. trichloroacetic acid-precipitable N/hr. For details of purification procedure see text.)

Fraction	Original homogenate	S_1	S_2	F_{50}	F_{65}
Activity	150	744	1128	1869	4520
Percentage of original cholinesterase content	100	81	33	26	24
μg. N/fraction derived from 1 ml. original homogenate	2390	446	119	51	19
Purification	—	×5	×8	×12	×30

bringing to pH 6·0 (glass electrode) a heavy protein precipitate developed, from which, on centrifuging, a clear brownish-yellow supernatant (S_2) could be decanted. Fraction S_2 was then treated with an equal volume of saturated ammonium sulphate solution, and the small precipitate which formed was discarded after centrifuging. The supernatant (F_{50}) was brought to 65 % saturation by the addition of solid ammonium sulphate; after standing for 10 min. the floccular precipitate containing the cholinesterase was thrown down by centrifuging. It was then dissolved in distilled water and dialysed overnight (16 hr.) against tap water. The small precipitate which formed at this stage was removed by filtering (Whatman no. 42 paper); the filtrate (F_{65}), which was a clear straw-coloured liquid, contained about 24 % of the total cholinesterase activity (estimated with acetylcholine as substrate) present in the original homogenate, and showed about a 30-fold purification when assessed as activity/mg. protein nitrogen. The degrees of purification obtained at the different stages are shown in Table 2.

Properties of the purified enzyme from heart. From our earlier experiments (Ord & Thompson, 1950) on the sensitivity to inhibition by DFP of the hydrolysis by rat heart of benzoylcholine and acetyl-β-methylcholine, it was concluded that the enzyme hydrolysing benzoylcholine was a pseudo-cholinesterase in the sense defined by Mendel & Rudney (1943). As confirmatory evidence of this it was decided to carry out more detailed studies of substrate specificity with various choline esters and with an aliphatic non-choline ester, tributyrin. A comparison of the results obtained with the unpurified homogenate (H) and with the partially purified, soluble cholinesterase (F_{65}) is given in Table 3, the relative rates of hydrolysis of the different substrates being expressed as percentages of the hydrolysis rate of acetylcholine. It will be seen that in the purified heart preparation the activity towards both acetyl-β-methylcholine and tributyrin is very significantly reduced, whereas the relative rates of hydrolysis of benzoyl-, propionyl- and butyryl-choline are almost unchanged. This finding supports the conclusion that at least two distinct esterases are present in these purified homogenates.

Table 3. *Relative rates of hydrolysis of choline esters and tributyrin by the original and the purified rat-ventricle preparations*

(Acetylcholine rates in each case taken as 100.)

	ACh	BCh	MCh	PrCh	BuCh	TB
Original preparation	100	29	9	227	138	70
Purified preparation (F_{65})	100	27	2	239	137	27

Summation experiments with mixed substrates were next carried out to obtain further evidence of the independence of the enzymes hydrolysing acetylcholine and tributyrin in this heart preparation, and also to discover whether acetyl-, propionyl- and butyryl-choline are each hydrolysed by the same enzyme. These results are summarized in Table 4.

It was found with the unpurified homogenate and also with the purified F_{65} fraction that the carbon dioxide output in the presence of tributyrin and either acetylcholine or butyrylcholine as the second substrate amounted to 86–107 % of the sum of the carbon dioxide output in the presence of each substrate separately, indicating that an 'ali-esterase' and a cholinesterase are present in the purified preparation. On the other hand, no summation of activity was obtained in the presence of acetylcholine and either propionyl- or butyryl-choline, indicating that these substrates are hydrolysed by the same enzyme.

The presence of a separate ali-esterase was confirmed by inhibition experiments in which it was found (Table 5) that concentrations of either eserine or DFP which inhibited completely or almost completely the cholinesterase, left the ali-esterase relatively unaffected.

The mean concentration of DFP required to cause 50 % inhibition of esterase activity (I_{50}) under our *in vitro* conditions was $0{\cdot}32 \times 10^{-8}$ M for the purified F_{65} fraction, as compared with $1{\cdot}4 \times 10^{-8}$ M for the original homogenate. This reduction in the I_{50} concentration for the purified as contrasted with the unpurified heart preparation probably reflects the removal of inert protein present in the whole homogenate, which had reacted with and thereby lowered the effective concentration of the DFP.

Table 4. *Hydrolysis rates of mixed substrates by rat-ventricle preparations*

(μl. CO_2/ml. preparation/30 min.)

1st ester	2nd ester		Both esters		'Summation'
TB	ACh	BCh	Found	Calculated	(%)
		Unpurified preparation			
114	157	—	233	217	86
114	—	59	158	173	91
PrCh					
323	150	—	263	473	0
TB		Purified preparation (F_{65})			
50	230	—	264	280	94
42	181	—	224	223	100
42	—	52	101	94	107
BuCh					
199	231	—	220	430	0

The above results obtained with a partially purified preparation of rat-heart ventricle confirm our earlier conclusion that this organ contains at least two enzymes hydrolysing choline esters, and reveal in addition the presence of an aliphatic esterase, hydrolysing tributyrin, but inactive against choline esters. The stages of purification have almost completely removed the true cholinesterase hydrolysing acetyl-β-methylcholine (Ord & Thompson, 1950), and have yielded a preparation

Table 5. *Effect of inhibitors on purified rat-ventricle preparations*

Inhibitor	Concn. (M)	Inhibition (%) ACh	PrCh	TB
Eserine sulphate	$0{\cdot}1 \times 10^{-6}$	80	—	0
Eserine sulphate	$1{\cdot}0 \times 10^{-6}$	96	—	0
DFP	$3{\cdot}5 \times 10^{-9}$	49	48	—
DFP	90×10^{-9}	100	—	32

containing an esterase highly active towards acetyl-, propionyl- and butyryl-choline. This second cholinesterase, however, although resembling the pseudocholinesterase present in many animal sera in its high sensitivity to inhibition by DFP and its inactivity towards acetyl-β-methylcholine, differs from it with regard to the optimal acyl group of choline esters hydrolysed by it; thus, it hydrolyses propionylcholine more rapidly than either acetyl- or butyrylcholine, differing, therefore, both from the cholinesterase of human or horse erythrocytes (Adams, 1949; Mounter & Whittaker, 1950) and from the cholinesterase of human or horse plasma (Adams & Whittaker, 1949*b*; Sturge & Whittaker, 1950).

Rat-brain cholinesterase

Effect of homogenizing medium on the extraction of brain cholinesterase. If homogenates of rat brain in 0·025 M-sodium bicarbonate are centrifuged at 3500–4000 rev./min. the greater part of the cholinesterase activity appears in the precipitate. Little (1948) has shown that the same is true for mouse brain, but found that if, instead of using sodium chloride-sodium bicarbonate mixtures, the brain was homogenized in distilled water, about 90% of the activity was present in the centrifuged supernatant; further, if sodium chloride solutions were used as the homogenizing medium the amount of enzymic activity in the supernatant decreased as the sodium chloride concentration was raised. Using human erythrocytes as their source of cholinesterase, Mentha, Sprinz & Barnard (1947) found that the enzyme can be extracted from the cells with ammonium hydroxide at pH 8·3, while Arragon & Sala (1948) succeeded in eluting it from kieselguhr with ammonia at pH 8·0–8·2. As a first step towards the purification of brain cholinesterase we therefore decided to study in greater detail the effect of differences in composition of the homogenizing medium on the proportion of cholinesterase extracted into the centrifuged supernatant.

Homogenates (10%, w/v) of whole rat brain were prepared in a variety of different aqueous solutions and in distilled water. The conditions of homogenizing and centrifuging were kept as far as possible identical. The pH of the homogenate was determined (glass electrode), and a sample taken for estimation of cholinesterase activity. The remainder was centrifuged for 10 min. at 4000 rev./min., and the activity of the supernatant estimated. The results of these experiments are shown in Table 6. No correlation was observed between the percentage of cholinesterase appearing in the supernatant and the pH of the medium. It will be seen, however, that those media, namely dilute sodium, ammonium or barium hydroxides or distilled water, in which a high proportion of cholinesterase is extracted into the supernatant, are all lacking in the physiological anions, Cl^- or HCO_3^-. The nature of the cations used, Na^+, K^+, NH_4^+, and Ba^{++} seemed insignificant.

Table 6. *Effect of homogenizing medium on the extraction of rat-brain cholinesterase*

(1 in 10 (w/v) homogenates used throughout; activity expressed as μl. CO_2/0·3 ml./30 min.)

Medium	pH of medium	Enzyme activity in: Whole homogenate	Enzyme activity in: Centrifuged supernatant	Percentage of total cholinesterase activity in supernatant
0·025 M-$NaHCO_3$	8·6	114	26	23
0·025 M-$KHCO_3$	8·6	123	21	18
0·025 M-NH_4Cl	5·1	154	46	30
0·9 % (w/v) NaCl	7·2	103	12	12
0·45 % (w/v) NaCl	7·1	117	17	15
0·025 M-$NaHCO_3$ + NaOH*	10·5	105	40	38
Glass-distilled water	6·0	101	74	74
NaOH*	10·6	87	76	87
NH_4OH*	10·5	104	98	94
$Ba(OH)_2$*	10·6	149	108	74

* Dilute NaOH, $Ba(OH)_2$ or NH_4OH added to water or, where stated, to 0·025 M-$NaHCO_3$ to bring to pH 10·5–10·6.

The dependence of the partition of the brain cholinesterase on the level of the anions Cl^- and HCO_3^- was confirmed by a series of experiments using water, dilute sodium bicarbonate, ammonium hydroxide, sodium hydroxide and sodium hydroxide/sodium bicarbonate mixtures as media in which the concentration of the brain in the homogenate ranged from 5 to 40 % (w/v) (Fig. 1).

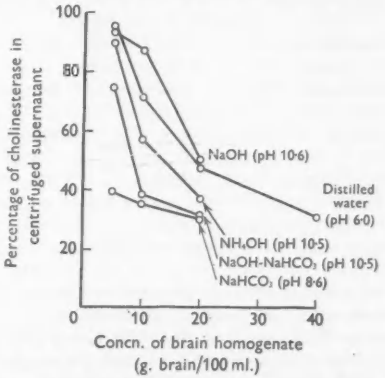

Fig. 1. Effect of dilution of brain homogenates on the degree of cholinesterase extraction.

The more concentrated brain homogenates will contain a higher concentration of Cl^- and HCO_3^- derived from the dispersed tissue, and it will be seen that with the concentrated preparations (20 %, w/v) none of the media extracted more than 50 % of the overall cholinesterase activity into the centrifugable supernatant. As the brain concentration is reduced, the anion concentration also falls, and the more dilute the preparation the greater is the percentage of the total cholinesterase present in the supernatant derived from water, NaOH or NH_4OH homogenates. The effect of the HCO_3^- ion is demonstrated strikingly, as it is only with the most dilute homogenate and in the presence of sodium hydroxide that a significant increase in the extraction of cholinesterase into the supernatant is obtained.

As a result of these experiments it was decided to use glass-distilled water (pH 6·0) as the homogenizing medium and a brain concentration of 10 % for the subsequent experiments.

Effect of surface-active agents on rat-brain cholinesterase. An initial problem that presents itself in the purification of cholinesterase from brain is the separation of an enzyme-containing protein fraction from the large amount of lipoid material present in this tissue. The supernatant (*W*) obtained by centrifuging homogenates of brain in water, even though it contains the greater part of the cholinesterase activity, is still opaque. In the hope of obtaining optically clear solutions on which to attempt further purification, the effect of the addition of various surface-active agents on these milky suspensions was next studied (Table 7).

The effect of these substances, present in a final concentration of 1 % (w/v), on the 'solubilization' of these brain supernatants was striking. Of the twenty compounds studied fifteen produced some degree of clarification of the milky supernatant, and eleven of them produced yellowish solutions which were almost optically clear. The degree of solubilization was largely independent of the ionic nature of the surface-active agent, although the non-ionic compounds appeared to be slightly less active. On the other hand, the different ionic types of agent produced very different effects on the cholinesterase activity of the preparation. None of the six non-ionic agents showed any inhibitory influence on the enzymic activity; indeed in each case slightly more than 100 % of the original activity was present. The four cationic agents and seven out of the ten anionic agents, however, caused an almost complete inhibition of the cholinesterase. 'Lissapol LS paste'

Table 7. *Effect of surface-active substances on the cholinesterase in centrifuged supernatants of water homogenates of rat brain*

(All surface-active agents present in final concentration of 1% (w/v). *N* = non-ionic, *A* = anionic, *C* = cationic. × × ×, rapidly and effectively cleared. × ×, cleared, more slowly; ×, slightly cleared.)

Surface-active agent	Ionic nature	Constitution	Degree of 'solubilization'	Cholinesterase activity as percentage original level
Perminal COL	*A*	Na salt of highly sulphonated oil, with fatty alcohol-polyethylene oxide condensate	× ×	1
Calsolene oil, HS	*A*	Highly sulphonated oil	× × ×	1
Dispersol L	*A*	Na salt of naphthalenesulphonate with H.CHO in aqueous solution	—	1
Sulphosal	*A*	Alkyl sulphate	× × ×	1
Teepol	*A*	Alkyl sulphate	× × ×	0
Perminal BX	*A*	Na salt of alkylated naphthalene-sulphonate	× × ×	3
Lissapol C paste	*A*	Sodium oleyl sulphate with water	× × ×	10
Lissapol LS paste	*A*	Sodium oleyl-*p*-anisididesulphonate with water	×	81, 77
Sodium cholate	*A*		× × ×	94, 86
Sodium deoxycholate	*A*		× × ×	53, 66
Cetrimide	*C*	Cetyltrimethylammonium bromide	—	0
Fixanol C	*C*	Cetylpyridinium bromide	× × ×	1
Fixanol VR	*C*	Tetradecylpyridinium bromide	× × ×	1
Lissolamine A50	*C*	Cetyltrimethylammonium bromide with water	—	2
Lubrol MO*	*N*	Fatty alcohol-ethyleneoxide condensate	—	101
Lissapol N	*N*	Polyoxyethylene condensate	×	114
Lissapol Nx	*N*	Anhydrous Lissapol N	×	108
Crill No. 6	*N*	Polyoxyethylene sorbitan monolaurate	—	112
Lubrol *W*	*N*	Cetyl alcohol-polyoxyethylene condensate	× ×	107
Saponin†	*N*		×	112

* Insoluble in water, therefore dispersed in supernatant. † Small amount added.

(sodium oleyl *p*-anisididesulphonate with water), sodium cholate and sodium deoxycholate, although causing some inhibition, did yield active preparations. As 'Lubrol *W*' (a cetyl alcohol-polyethylene oxide condensate) was the most effective of the non-ionic substances as regards clarification, highly translucent preparations being obtained with it, further work was carried out with this compound and also with cholic and deoxycholic acids. It was shown that 1% Lubrol *W* did not affect the rates of hydrolysis of acetylcholine, benzoylcholine or acetyl-β-methylcholine, nor the degree of inhibition produced by DFP.

Purification of brain cholinesterase: (*a*) *In the absence of surface-active agents.* Various attempts were made to purify the supernatant (*W*) obtained after homogenization in distilled water. Fractional precipitation by increasing the acidity resulted in insoluble but fully active precipitates (at about pH 5·5), and it was not possible to obtain a clear supernatant with appreciable activity as in the case of the rat-heart preparations. Fractional precipitation with ammonium sulphate yielded three distinct protein fractions: (1) precipitated at 7% saturation, and containing 58% of the cholinesterase activity of the whole fraction *W*; (2) precipitated at 23% saturation, and (3) a fraction precipitated at 46% saturation. Both these last fractions contained small amounts of cholinesterase activity.

The precipitate obtained at 7% saturation (P_7) was washed once with water to remove excess ammonium sulphate, and could then be resuspended to give a stable white suspension in water. On bringing to pH 5·5 a floccular precipitate formed; on resuspending this in water and enzymic activity was fully recovered; on bringing to pH 12 the precipitate dissolved completely, but was irreversibly inactivated. The neutral aqueous sus-

pension of P_7 was also soluble in the presence of 0·5 % Lubrol *W*.

This solution retained its full cholinesterase activity, but, in contrast to the stable suspension without Lubrol *W*, was completely inactivated on bringing to pH 5·5. 0·3 % Sodium deoxycholate behaved similarly, yielding solutions containing 60 % of the original cholinesterase activity, the solution again being inactivated by hydrochloric acid at pH 5·5.

The effect of short periods of digestion with proteolytic enzymes (trypsin, pepsin, papain) on P_7 was also studied; although trypsin caused some measure of solution of the suspension, all three proteinases inactivated the preparation. The neutral aqueous suspension of fraction P_7 showed an activity of 2480 μl. carbon dioxide/mg. total nitrogen/hr., compared with an activity of 280 μl. carbon dioxide/mg. total nitrogen/hr. for the original homogenate, i.e. a ninefold purification is achieved by this single ammonium sulphate precipitation. We next attempted the purification of homogenates previously treated with surface-active agents.

(*b*) *In the presence of* 1 % *Lubrol W*. On treatment of the milky supernatant (*W*) from the centrifuged water homogenate of rat brain with 1 % Lubrol *W* translucent yellowish solutions were obtained. On adjusting the pH to 4·5 a bulky precipitate formed. After centrifuging a clear yellow supernatant was obtained, containing 39 % of the original cholinesterase activity towards acetylcholine. This solution was then brought to pH 8·0, and saturated ammonium sulphate solution added to about 24 % saturation, when the first permanent cloudiness appeared. The preparation was filtered (Whatman no. 42), the precipitate discarded, and the filtrate brought to 50 % saturation. The precipitate which now formed separated on top of the centrifuged solution, and, after decanting the latter, was found to be immediately soluble in water. After dialysis overnight a yellowish solution (*SB*) was obtained, which contained 25 % of the original cholinesterase activity, and was ten times purer than the original homogenate when assessed on mg. total nitrogen basis.

Attempts at further purification of this preparation by adsorption or reprecipitation were unsuccessful.

Properties of purified preparations of brain cholinesterase. Substrate specificity studies with acetylcholine, benzoylcholine, acetyl-β-methylcholine and tributyrin have been carried out on the purified fraction *SB* derived from a Lubrol-treated water homogenate of brain. Table 8 shows the relative rates of hydrolysis of these substrates expressed as percentages of the hydrolysis rate of acetylcholine.

It will be seen that while the original brain homogenate readily hydrolysed tributyrin, the purified fraction *SB* did not, although retaining its full activity towards acetyl-β-methylcholine. The level of hydrolysis of benzoylcholine is too low to permit any conclusion as to whether this compound is hydrolysed by the acetylcholine-hydrolysing enzyme or not.

Table 8. *Relative rates of hydrolysis of choline esters and tributyrin by a whole homogenate of brain and by the soluble, purified fraction SB*

(ACh rates in each case taken as 100.)

	ACh	BCh	MCh	TB
Whole homogenate	100	6	75	44
Fraction *SB*	100	4	81	0

The evidence from the above experiments for the presence of a distinct enzyme hydrolysing tributyrin is confirmed by the finding that whereas 1 % Lubrol *W* has no effect on the cholinesterase activity of these brain homogenates, it produces about 40 % inhibition of hydrolysis of tributyrin.

DISCUSSION

As stated earlier, the purpose of this work was to obtain soluble preparations of true and pseudocholinesterases from innervated tissues. Since the highest centrifuging speed which we had available was 4000 rev./min. no claims can be made that the cholinesterase present in the centrifuged supernatants of water homogenates of brain is in true colloidal solution; it is very possible that it is still attached to small particles which would sediment at slightly higher speeds of centrifugation. Our final preparations from heart (F_{65}) and from brain (*SB*), however, were obtained by the further purification of optically clear solutions, which had themselves been obtained by the previous removal of heavy protein precipitates first by acidification and subsequently by salting-out. As they have not been tested by high-speed centrifugation it is not possible to state that their contained proteins are fully dispersed. From the earlier precipitation stages and the physical appearance of the final preparations (F_{65} and *SB*), however, we think it reasonable to conclude that we have obtained colloidal solutions of these cholinesterases, although in the case of brain a 'solubilizing' agent is present so that we cannot exclude the possibility that our solution may contain relatively large solubilized lipoprotein or other complexes. Although no high degree of purification of these enzymes has been achieved (30-fold for the heart cholinesterase, and 10-fold for the brain), the partially purified, soluble preparations reveal certain further differences among the tissue cholinesterases, apart from the well known difference in their activity towards benzoylcholine and acetyl-β-methylcholine.

For example, the high rate of hydrolysis of propionylcholine by the purified preparation from rat heart indicates that there is a cholinesterase which belongs neither to the aceto-cholinesterase nor to the butyro-cholinesterase types described by Sturge & Whittaker (1950); while such a classification on grounds of substrate specificity evidently holds for the enzymes in the three tissues (brain, erythrocytes and plasma) studied by these workers, it would seem that we have not yet sufficient evidence to generalize from these cases to the cholinesterases present in other tissues of the body.

The cholinesterases of different tissues may in fact comprise a group of related enzymes showing a range of differences in their specificity towards artificial substrates. Thus, in addition to this enzyme in rat heart which differs from others so far studied in respect of its activity towards propionyl choline, Sawyer (1945) has described a benzoylcholinesterase, and Levine & Suran (1950) have recently reported a further type of cholinesterase in swine serum which is inactive towards both benzoylcholine and acetyl-β-methyl-choline.

A further point of difference is shown by the relative resistance of the heart and brain enzymes to digestion by trypsin; while the cholinesterase in rat heart which is active against benzoylcholine is not affected by short periods of tryptic digestion of the crude tissue homogenate, the cholinesterase in brain is inactivated by such treatment. That this is not a peculiarity common to so-called true cholinesterases, however, is shown by the fact that in further preliminary experiments we have found that the cholinesterase in rat diaphragm can be extracted unimpaired by tryptic digestion.

The effect of surface-active agents on brain cholinesterase does not appear to have been studied hitherto, although Huidobro & Atria (1949) have reported on the effects of five different detergents on human serum cholinesterase; all of the compounds studied by them were ionic, and only one, 'Zephiran', a high-molecular alkyldimethylbenzylammonium chloride, had any effect on cholinesterase activity, causing only approximately 50% inhibition under their conditions. Their results with the serum enzyme seem to be in striking contrast to those obtained here with the brain enzyme, in which eleven out of fourteen ionic surface-active substances caused a complete or almost complete inhibition of activity. Rossiter (1949) has also used surface-active substances (saponin, 'alkyl sulphate' and bile salts) to liberate the esterase from rabbit polymorphonuclear leucocytes, and has found that none of these substances inhibits this enzyme.

The action of these surface-active agents in bringing into solution the brain cholinesterase raises the question of the mechanism of this effect. It is unlikely that it is a liberation of the enzyme from the brain cells, as in the lysis of red blood cells or in the less drastic action of detergents on intact polymorphonuclear leucocytes (Rossiter, 1949), since our starting material was a centrifuged supernatant of water homogenates of brain, in which, presumably, the larger formed elements will no longer be present. It is therefore more probably a true solubilizing effect on lipoprotein complexes suspended in solution and containing the cholinesterase. It should be pointed out that not only are the centrifuged homogenates grossly opaque unless clarified by a surface-active substance, but that the partially purified precipitate, P_7, salted out with only 7% saturated ammonium sulphate, is also insoluble in water at pH 7·0 unless Lubrol *W* or sodium deoxycholate is present. We think that these findings are consistent with the view that the brain cholinesterase may itself be a lipoprotein, or that it occurs in intimate association with other lipoprotein complexes. If this should be true it may be expected to influence the relative ease of penetration of different cholinesterase inhibitors to the enzymes in brain and, for example, in serum, quite apart from any differences of affinity that such inhibitors may have for the active centres of the proteins in question.

SUMMARY

1. Soluble preparations of cholinesterases from rat heart and brain have been obtained, the enzyme from heart being purified approximately 30-fold and the brain enzyme approximately 10-fold.

2. Specificity and inhibition studies on the heart preparation have revealed the presence of at least two cholinesterases and an ali-esterase. The cholinesterase present in greatest amount exhibits certain differences from both the true and pseudo-cholinesterases described hitherto.

3. A study of the effect of twenty surface-active substances on the extraction and activity of rat-brain cholinesterase has been made. Out of fourteen different ionic surface-active substances eleven caused a complete or almost complete inhibition of activity. All six non-ionic substances left the activity unimpaired.

4. The mechanism of the 'solubilizing' action of certain of these surface-active agents on the brain enzyme is discussed.

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REFERENCES

Adams, D. H. (1949). *Biochim. Biophys. Acta*, **3**, 1.
Adams, D. H. & Whittaker, V. P. (1949*a*). *Biochem. J.* **44**, 62.
Adams, D. H. & Whittaker, V. P. (1949*b*). *Biochim. Biophys. Acta*, **3**, 358.
Ammon, R. (1933). *Pflüg. Arch. ges. Physiol.* **233**, 486.
Arragon, G. & Sala, E. (1948). *Bull. Soc. Chim. biol., Paris*, **30**, 44.
Huidobro, E. & Atria, P. (1949). *J. Pharmacol.* **96**, 438.
Levine, M. G. & Suran, A. A. (1950). *Nature, Lond.*, **166**, 698.
Little, J. M. (1948). *Amer. J. Physiol.* **153**, 436.
McCance, R. A. (1950). Private communication.
McMeekin, T. L. (1939). *J. biol. Chem.* **128**, lxvi.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Mendel, B. & Mundell, D. B. (1943). *Biochem. J.* **37**, 64.
Mendel, B. & Rudney, H. (1943). *Biochem. J.* **37**, 59.
Mentha, J., Sprinz, H. & Barnard, R. (1947). *J. biol. Chem.* **167**, 623.
Mounter, L. A. & Whittaker, V. P. (1950). *Biochem. J.* **47**, 525.
Nachmansohn, D. & Rothenberg, M. A. (1945). *J. biol. Chem.* **158**, 653.
Nachmansohn, D. & Rothenberg, M. A. (1947). *J. biol. Chem.* **168**, 223.
Ord, M. G. & Thompson, R. H. S. (1950). *Biochem. J.* **46**, 346.
Rossiter, R. J. (1949). *J. Physiol.* **110**, 136.
Sawyer, C. H. (1945). *Science*, **101**, 385.
Stedman, E. & Stedman, E. (1935). *Biochem. J.* **29**, 2563.
Strelitz, F. (1944). *Biochem. J.* **38**, 86.
Sturge, L. M. & Whittaker, V. P. (1950). *Biochem. J.* **47**, 518.

The Relative Metabolic Inertia of Tendon Collagen in the Rat

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The protein collagen, various polysaccharides and other substances have been known for many years to be present in the extracellular spaces of the body. Changes in these tissue components in disease have been demonstrated by pathologists, and recent clinical and anatomical studies on rheumatism and allied diseases have greatly stimulated further work on the 'ground substances' of the extracellular spaces. Very little is known, however, about the metabolism of these substances even in the normal subject. Information, therefore, on the metabolism of collagen would seem to be of interest. This protein is widely distributed throughout body tissues and is found in large amounts in bone, cartilage, tendons and skin. At an estimate, about one-third of the total mass of the protein of the mammalian body consists of collagen and it is thus important to establish whether this extracellular protein is, like most intracellular proteins, constantly broken down and resynthesized, even in the adult animal. Collagen is particularly rich in glycine, and glycine labelled with ^{14}C in the methylene group was therefore used to determine the turnover of collagen in three series of rats. For preliminary communication, see Perrone & Slack (1951).

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EXPERIMENTAL

General

Treatment of animals and administration of labelled glycine. Male albino rats of the Institute stock were used throughout. Series *A* consisted of four rats with body weight ranging from 358 to 390 g.; their ages were not known exactly, but all animals of this group were more than 14 months old. Series *B* consisted of five young adults (initial body wt. 203–207 g.), whilst series *C* consisted of five young rats with initial body weight ranging from 50 to 80 g. All animals were apparently in normal health and had not been used for other experiments. Throughout the experiment they were given the Institute stock diet. The labelled glycine, which was kindly supplied by Dr H. R. V. Arnstein, had an activity of 1 μc./mg.; the required dose was mixed with about 30–50 mg. non-isotopic glycine and fed in the diet over a period of 3 days at the beginning of each experiment. The rats of groups *A* and *B* each received 10 μc./100 g. initial body weight. Three of the rats of group *C* (nos. 99, 100 and 104) received 40 μc./100 g. initial body weight, whilst the other two (nos. 115 and 116) received 6·25 μc./100 g. initial body weight. The animals were killed by stunning at varying times during the experiment.

Growth of animals. The old rats of group *A* showed no marked or consistent change in body weight (Table 1). The young adults of series *B* showed a steady increase of weight during the experiment, the rate varying somewhat with different animals (Table 2). The young rats of series *C* increased their body weight very markedly during the experi-

ment (Table 3). Since this matter is of considerable importance for the interpretation of the isotope results the actual growth curves of the last group are shown in Fig. 1.

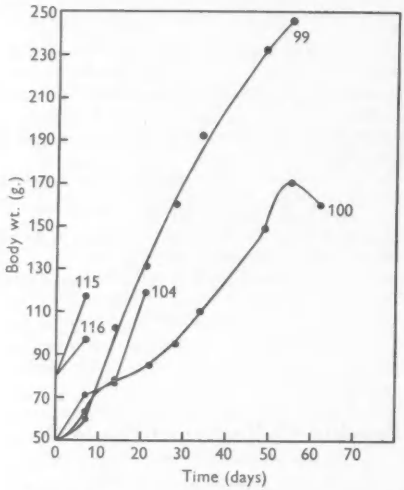

Fig. 1. Growth curves of the young rats of series *C* (Table 3).

Preparation of samples

Liver and muscle. The livers and representative muscles from the four limbs were removed and finely minced. The samples were spread around the inner walls of flasks of 250 ml. capacity, with the aid of a little distilled water and freeze-dried. Drying was begun within 3 hr. of killing the animal. Free glycine in tissues was removed by repeated extraction of the dry samples with boiling 75% (v/v) ethanol; the samples were then defatted by ether extraction. Hydrolysis was carried out by boiling, under reflux, suspensions of the dry material (2 g. approx.) in 6N-HCl (approx. 100 ml.) for 48 hr. The solutions were taken to dryness and the residue dissolved in water. Treatment with 1:2:4-fluorodinitrobenzene was carried out by a slight modification (Perrone, 1951) of the method of Sanger (1945).

Collagen. The collagen studied in this investigation was all obtained from white tendons. These were dissected free of muscle fibre and fascia, and consisted of a mixed sample of about 1 g. (fresh weight) of cleaned tendon obtained from all four limbs, the back and tail of the rat. The method of purification was similar to that of Bowes & Kenten (1948) with the following additional procedure. After thorough cleaning by alternate and repeated extractions with distilled water and 10% (w/v) NaCl the collagen was refluxed with approx. 0·1% acetic acid (50 ml.) overnight. The residue of elastin was filtered off. For determinations of dry weight, the gelatin was precipitated by careful neutralization of the filtered acetic acid solution with ammonia, followed by addition of acetone (950 ml.). The solid material was centrifuged off and dried *in vacuo* over P_2O_5. The purification of the collagen from some tissues presents difficulties (Lowry, Gilligan & Katersky, 1941), but rat tendon appears to yield pure collagen readily. The collagen obtained by the above procedure gave negative reactions for aromatic amino-acids and showed no selective absorption between 257·5 and 287·5 mμ. (Loofbourow, Gould & Sizer, 1949). Further evidence of purity is the very low specific activity found in the glycine obtained by hydrolysis of the collagen of the tendons of the old rats.

The purified gelatin from 1 g. fresh white tendons was hydrolysed by refluxing with 6N-HCl (50 ml.) for 24 hr. The solution was reduced to dryness, the residue taken up in water and the 2:4-dinitrophenyl (DNP) amino-acids prepared as described above.

Isolation of DNP-glycine. Glycine from the hydrolysates of liver, muscle and collagen was separated as the DNP derivative on Celite columns containing M-NaH_2PO_4 as the stationary phase and 10% (v/v) ether in $CHCl_3$ as the mobile phase. Rapid and effective separation of glycine is achieved by this method (Perrone, 1951). From the hydrolysate of 1 g. fresh tendon, amounts of crystalline DNP-glycine greater than 50 mg. were always obtained using a column containing approx. 50 g. Celite 545. Crystallization was effected by dissolving material obtained from the column in dioxan and adding hot *cyclo*hexane. DNP-glycine so obtained appeared to be pure as shown by chromatographic behaviour, melting point which was 205° (uncorr.) and mixed melting point.

Radioactivity measurements

The specific radioactivities of the DNP-glycine samples were determined with a bell-shaped helium-filled Geiger-Müller counter having a thin mica window. The counter has an unusually low background count of 11–13 counts/min. The measurements were carried out on solid samples placed on disks of identical geometry and 2 sq.cm. area. The material was spread evenly by the pellet technique (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949; Popják and Beekmans, 1950). Each sample contained at least 25 mg./sq.cm.; under such conditions of infinite thickness, the number of counts is linearly proportional to activity. No correction for self-absorption was made. Results are expressed as counts/min./2 sq.cm.

RESULTS

Changes in the specific activities of glycine obtained from the mixed proteins of liver and muscle

The glycine obtained from liver protein showed a very high activity 24 hr. after the feeding of the labelled amino-acid had been completed. This activity decreased very rapidly over the following 35–50 days (Tables 1–3). In the young adults (Table 2) the isotope content decreased over the first 3 weeks by a factor of approx. 2·5/week, but it appears from the rather scanty data that the activity-time curve of the liver glycine flattened out later. In the young rats (Table 3) the activity fell more sharply with time than in the older animals. This is presumably associated with the rapid rate of growth which was found with these animals. This point will be discussed later.

The radioactivity of the glycine obtained by hydrolysis of the mixed proteins of muscle showed,

Table 1. *Specific radioactivities of* 2:4-*dinitrophenylglycine samples obtained from liver, muscle and tendon collagen of old rats fed* $NH_2{}^{14}CH_2.COOH$

Rat no.	Body weight Initial (g.)	Body weight Final (g.)	Dose of ^{14}C-labelled glycine/100 g. body wt. (μc.)	Duration of experiment (weeks)	Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of: Liver	Muscle	Collagen
101	373	355	10	7	184*	348*	15
102	385	380	10	7			—
105	358	371	10	3	466	454	24
106	390	370	10	3	505	493	12

* Pooled samples.

Table 2. *Specific radioactivities of* 2:4-*dinitrophenylglycine samples obtained from liver, muscle and tendon collagen of young adult rats fed* $NH_2{}^{14}CH_2.COOH$

Rat no.	Body weight Initial (g.)	Body weight Final (g.)	Dose of ^{14}C-labelled glycine/100 g. body wt. (μc.)	Duration of experiment (days)	Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of: Liver	Muscle	Collagen
1	234	237	10	1	3700	698	46
2	233	242	10	7	1414	603	68
3	203	229	10	14	550	422	50
4	206	252	10	21	286	454	127
5	205	236	10	35	148	383	151

Table 3. *Specific radioactivities of* 2:4-*dinitrophenylglycine samples obtained from liver, muscle and tendon collagen of young rats fed* $NH_2{}^{14}CH_2.COOH$

Rat no.	Body weight Initial (g.)	Body weight Final (g.)	Dose of ^{14}C-labelled glycine/100 g. body wt. (μc.)	Duration of experiment (weeks)	Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of: Liver	Muscle	Collagen	Activities (counts/min./2 sq.cm. disk, infinite thickness) corrected for dose ^{14}C of 10 μc./100 g. body weight: Liver	Muscle	Collagen
115	79	115	6·25	1	722*	594*	428	1155*	950*	685
116	80	97	6·25	1			376			601
104	50	119	40	3	592	799	947	148	200	237
99	50	246	40	7½	88	308	526	22	77	131
100	50	160	40	8½	—	—	773	—	—	193

* Pooled samples. — Means not determined.

like that of liver, a steady fall with time in all three groups of rats (Tables 1–3). In group *B* the initial value was only about 20 % of that of the glycine of the liver, but the rate of decrease was very much slower so that after 2–3 weeks the activity of the glycine of muscle equalled that of the glycine of liver, and with animals killed 35 days or more after the beginning of the experiment the activity of the former always exceeded that of the latter by a large amount. The specific activity of the glycine from muscle protein like that from liver protein, decreased more sharply in rapidly growing animals than in adults.

Changes of specific activity of the glycine obtained from the collagen of the tendons

The glycine isolated from the collagen of old rats after 3 and 7 weeks showed a very low radioactivity (Table 1). In young adults the activity was slightly higher, but still very low, compared with that of the glycine of muscle or liver protein. Unlike the latter, the activity of the glycine of the collagen increased slowly during the experiment (Table 2). With the very young rats, on the other hand, initial activity was very high, only slightly lower than the corresponding value for muscle and about half that for liver (Table 3). In this experiment the activity decreased with time in marked contrast to the young adults.

Effect of growth on the changes of specific activity with time

The young rats used in the third series grew rapidly during the experiment, whilst those of the second series were older and grew only at a low rate. The changes in body weight of the old rats were small and probably due entirely to changes in fat or water content of the body. The considerable differences

found between the three groups of rats especially with respect to collagen, can be entirely explained in terms of growth. During the period while the labelled glycine is being fed and for a short time afterwards, glycine with a high isotope content is incorporated into body proteins. Later, however, the radioactivity of the glycine available for protein synthesis decreases rapidly and, at least in the rat, becomes negligible within a few days compared with the initial radioactivity. If the newly formed protein is metabolically inert like the haemoglobin of the red cell, the radioactivity of the protein will then become constant, provided no growth takes place. If, however, the net amount of the protein increases throughout the experiment, the initial radioactivity will fall, owing to dilution by deposition of new protein of rapidly decreasing isotope content. If the protein under investigation is not metabolically inert, this fall of activity, which is specifically associated with growth, will be superimposed on the decrease of isotope content which is caused by the workings of normal catabolic and anabolic processes. For a correct evaluation of this 'growth dilution' effect, it would be necessary to know the exact increase in the particular protein and also the changes of specific radioactivities of the glycine used for protein synthesis throughout the experimental period. Such data do not exist, and we have therefore assumed that the net increases in muscle protein, liver protein and collagen are linearly proportional to increase in body weight. We have further assumed that the isotopic material is introduced into the body over an infinitesimally short period at the beginning of the experiment and that the isotope introduced into the protein later can be neglected. The specific activities found were therefore multiplied by the ratio of final to initial body weight and the corrected values thus calculated are shown in Table 4. This very simplified treatment is unlikely to give accurate results, but may yield an indication of the order of magnitude of this effect of 'growth dilution' on activity-time curves.

Table 4 shows that the rate of decrease of activity of the glycine of liver and muscle protein is more or less the same in all three groups of rats, i.e. the apparently greater decrease in young rats as compared with older animals, can be ascribed to a net increase of protein involving dilution with material of low isotope content. The activity of the glycine of collagen, on the other hand, decreases either not at all or only slightly in these young rats and the decrease observed in the uncorrected values is almost entirely due to 'growth dilution'.

DISCUSSION

The turnover rates of liver and muscle proteins

Schoenheimer, Rittenberg and their colleagues, using amino-acids labelled with ^{15}N, have carried out extensive investigations on the turnover rates of the mixed proteins of various organs of the rat (see, for example, Ratner, Rittenberg, Keston & Schoenheimer, 1940; Shemin & Rittenberg, 1944). Our data on liver and muscle extend somewhat the results of the American workers. The data on the specific activities of the glycine obtained from the mixed liver proteins of the young rats fit, apart from the last figure, reasonably well the equation

$$\ln i_t = A - kt,$$

where i_t is the activity (in counts/min.), t the time (in days) and A and k constants. The best values for A and k are 8·36 and 0·145, respectively. From this the half-life $t_{20\cdot5}$ can be calculated to be about 4–5 days. Values obtained later than 21 days do not fit a logarithmic curve and the activities are higher than expected. This is probably partly caused by the heterogeneous character of the liver proteins; collagen in particular with its high glycine content may retain the labelled amino-acid for long periods

Table 4. *Results of experiments (Tables 1, 2 and 3) corrected for increase in body weight and reduced to a dose of 10 μc./100 g. body weight, expressed as counts/min./2 sq.cm. disk, infinite thickness*

(For details of the correction, see text.)

Time (days)	Old rats			Young adults			Young rats		
	Liver	Muscle	Collagen	Liver	Muscle	Collagen	Liver	Muscle	Collagen
1	—	—	—	3700	698	46	—	—	—
7	—	—	—	1470	626	70	1530	1188	852
14	—	—	—	623	478	58	—	—	—
21	505 466	493 454	12 24	350	555	159	391	480	564
35	—	—	—	170	441	174	—	—	—
49	184	348	15 17	—	—	—	—	—	—
53	—	—	—	—	—	—	109.	379	645
60	—	—	—	—	—	—	—	—	618

— Means not determined.

and cause a tailing of the activity-time curve. Another complicating factor is the reversible transfer of amino-acids between tissues of high turnover rate such as liver and those of low turnover rate, like muscle, a point already emphasized by Shemin & Rittenberg (1944). Since muscle has a higher activity than liver in the later stages of the experiment, such a transfer of labelled glycine would reduce the rate of decrease of isotope in muscle in the early stages of the experiment and in liver in the later stages.

The data for muscle fit a logarithmic curve rather badly, but very approximate values for k can be calculated. These are between 0·023 and 0·029, corresponding to half-life periods of 24–30 days. It is probable, and this is also suggested by unpublished observations of Sprinson & Rittenberg (1949), that muscle proteins are metabolically very heterogeneous and average half-life figures have therefore not much theoretical significance in this case. It is of interest that the activity-time curves of muscle and liver glycine cross at about 20 days. The fact that the activity of the sample isolated after 5 weeks was only 40 % lower than that found after 1 day (Table 2) shows that the radioactive glycine is retained in the muscular tissue for a relatively long time. Shemin & Rittenberg (1944) analysed glycine obtained from the combined skin, muscle, tendons, etc., and found a steadily increasing isotope content. Since their 'carcass' fraction contained considerable amounts of collagen and since their rats were still growing and therefore deposited new collagen, the observed increase in isotope content was almost certainly due to the glycine derived from collagen.

The turnover of tendon collagen

All the results reported in this paper are compatible with the assumption that collagen, once it is deposited in the intercellular spaces, becomes metabolically inert and is, if at all, only very slowly degraded in the normal animal. That in the young rat dietary glycine is utilized by the fibroblasts for collagen formation is shown by the high initial activities found in the rapidly growing rats and the moderate activities observed in the slowly growing young adults. The reasons for the decrease in specific activity of the collagen glycine in the young rats have already been discussed, but it remains to consider the causes for the steady increase found with the young adults. In growing rats there are two processes which oppose each other in their effects on activity changes in the collagen. On the one hand, the glycine circulating in the body and available for protein synthesis after the feeding of the labelled compound has been discontinued, has a significant, but rapidly diminishing radioactivity and the total isotope content of the glycine of the collagen is bound to increase, provided the deposited collagen is metabolically inert. But, if the animal grows rapidly, the rate of increase of total collagen may be greater than the rate of increase of total radioactivity. In such a case the specific activity of the glycine will decrease and so explain the fall found with the uncorrected values of young rats (Table 3). If, on the other hand, growth is slow, the specific activity of the newly formed collagen may be higher than that of the collagen already deposited and thus specific activity will increase until the activity of the 'free glycine' of the body has decreased to that of the glycine residue in collagen. This would explain the rise of activity in the slowly growing young adults. The very slight activities found in the old rats (Table 1) cannot be explained by general growth, but it is likely that the collagen in certain organs increases in these old animals. Thus the collagen content of rat muscle (Lowry, Hastings, Hull & Brown, 1942) and also that of the aorta (Myers & Lang, 1946) increases appreciably on ageing.

But the present results by no means exclude the possibility that the collagen of the tendon participates to some extent in the dynamic processes of the body. In old rats the turnover rate must be extremely small, corresponding to a half-life period of several hundred days and of a similar order of magnitude as the normal life span of the rat. With young rats, such a turnover may be somewhat faster and the fact that the 'corrected' isotope contents of the young rats (Table 4) do not increase with time, like those of the young adults, indeed suggests that the collagen of the tendon is not completely inert in very young rats. The possibility that collagen changes in its metabolic behaviour with the age of the animal is suggested by the physical differences which have been observed (Lloyd & Marriott, 1935; Leplat, 1935) in collagen samples obtained from the tendons of young and old animals respectively.

It is impossible with the data available to decide as to which of the alternative interpretations is correct. But in any case the present results clearly indicate that the collagen of the rat tendon is metabolically inert relative to the mixed proteins of liver, other internal organs and even of muscle. It would be unjustified to assume that the collagens of the skin and bone behave in a manner identical with that of the tendon, but preliminary experiments indicate that the turnover rate of skin collagen is at least quite low. It is therefore probable that all extracellular proteins are very much less active metabolically than the intracellular proteins which predominate in liver, kidney, etc., and also in muscle. Sprinson & Rittenberg (1949) have emphasized recently the limitations of the concept of the dynamic state of body constituents as applied to some of the proteins of man and to a lesser extent to those of the rat. If the generalization is correct, that all extracellular proteins resemble collagen of the tendon, it would

follow that about 40 % of the total protein of the rat does not participate to an appreciable extent in the reversible and balanced processes which characterize the dynamic state of the body. It would also imply that about 75 % of the total glycine, a large part of the proline and almost all the hydroxyproline in the normal adult rat are in a static state.

The present results have some bearing on the question of health hazards involved in handling radioactive compounds which may become incorporated into the body proteins. Ingestion of radioactive glycine, for example, will result in high initial radioactivities in the proteins of the liver, intestine and organs of similar metabolic type. However, owing to the rapid metabolism of the proteins of these organs, such high activities will only be maintained for short periods, unless exposure is prolonged. The labelled compound is likely to be incorporated into muscle proteins to a smaller extent than into the liver proteins, but will be retained for relatively long periods. No appreciable entry into the collagen of the adult is likely to occur, but in the young growing subject the radioactive compound may become fixed in the extracellular proteins for a very long time. This would constitute a serious potential hazard peculiar to the child and adolescent.

SUMMARY

1. Glycine labelled with ^{14}C in the methylene position was fed to three series of rats, old rats, young slowly growing adults and rapidly growing young ones. The animals were killed at varying intervals of time and samples of liver, muscle and of collagen from white tendons were obtained. 2:4-Dinitrophenylglycine from these three sources was crystallized after chromatographic separation and the radioactivity determined.

2. The specific activity of glycine from liver protein showed a very high initial activity which decreased rapidly during the subsequent 35–50 days. The results indicate that the half-life of the mixed liver proteins is 4–5 days. With muscle the initial activity was lower than in liver and there was a steady but slow fall with time. After 2–3 weeks the activity of muscle glycine equalled that of liver and later exceeded it.

3. The glycine from the collagen of old rats showed a very low radioactivity. In young adults the activity was slightly higher, but still very low compared with that of the glycine of muscle or liver. There was a slow increase of activity with time. With the very young rats initial collagen activity was very high, only slightly lower than the corresponding value for muscle and about half that of liver. The activities found in the young rats decreased with time in marked contrast to the young adults.

4. The results indicate that tendon collagen in the old rats is metabolically almost completely inert. In young rats interpretation is complicated by growth, but when correction is made for this factor it appears that collagen is relatively inert even in young actively growing animals, although the possibility cannot be excluded that the turnover rate of collagen is somewhat faster than in older animals.

5. It is pointed out that radioactive compounds may be deposited in the extracellular spaces of young subjects where they may remain for long periods.

REFERENCES

Bowes, J. H. & Kenten, R. H. (1948). *Biochem. J.* **43**, 358.

Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. F. (1949). *Isotopic Carbon*, New York: Wiley.

Leplat, G. (1935). *Arch. Biol., Paris*, **46**, 339.

Lloyd, J. D. & Marriott, R. H. (1935). *Proc. roy. Soc.* B, **118**, 439.

Loofbourow, J. R., Gould, J. R. & Sizer, I. W. (1949). *Arch. Biochem.* **22**, 406.

Lowry, O. H., Gilligan, D. R. & Katersky, E. M. (1941). *J. biol. Chem.* **139**, 795.

Lowry, O. H., Hastings, A. B., Hull, T. Z. & Brown, A. N. (1942). *J. biol. Chem.* **143**, 271.

Myers, V. C. & Lang, W. W. (1946). *J. Gerontol.* **1**, 441.

Perrone, J. C. (1951). *Nature, Lond.*, **167**, 513.

Perrone, J. C. & Slack, H. G. B. (1951). *Biochem. J.* **48**, iv.

Popják, G. & Beckmans, M. L. (1950). *Biochem. J.* **46**, 547.

Ratner, S., Rittenberg, D., Keston, A. S. & Schoenheimer, R. (1940). *J. biol. Chem.* **134**, 665.

Sanger, F. (1945). *Biochem. J.* **39**, 507.

Shemin, D. & Rittenberg, D. (1944). *J. biol. Chem.* **153**, 511.

Sprinson, D. B. & Rittenberg, D. (1949). *J. biol. Chem.* **186**, 715.

'Apparent Protein' in Human Urine: some Surface Film Measurements

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The traces of protein-like substances present in normal urine are rarely detected by routine laboratory tests. Surface-tension methods, widely used in protein chemistry, are more sensitive. Such a method was first applied to the determination of urinary protein by Gunton & Burton (1947), who added diluted urine to an aqueous buffer solution at pH 6·4 and measured the area of the surface film at 9 dynes/cm. using an indicator drop. They reviewed the literature and recorded 3·7 mg./100 ml. as the mean protein content of normal urine. A study of urinary proteins in tuberculosis patients made it desirable to extend this work. The first departures from Gunton & Burton's technique were made to fit the available apparatus, and further simplifications were introduced. This modification of the method is described. Since neither Gunton & Burton's method nor the present procedure involves purifying or characterizing the urinary proteins, the term 'apparent protein' is used here to denote the resultant of all surface-active substances present in undialysed urine. Fluctuations in the 'apparent protein' content of consecutive urine specimens were also determined in some normal and tuberculosis cases. Only sufficient data were collected to define the usefulness of the method, but the results are of interest on their own merit and are presented here.

EXPERIMENTAL

Equipment. A rectangular trough, liquid surface 331 × 182 mm., and two rigid barriers were used. These were all made of Perspex and were coated with paraffin which was frequently renewed. The pH 6·4 buffer was prepared as described by Gunton & Burton (1947) and was preserved with $CHCl_3$. Technical White Oil was used as an indicator. A sample of this, darkened by heating in air, was mixed with unheated White Oil until the mixture, tested under experimental conditions, gave a convenient reading with a specimen of normal urine. The same oil was used throughout the work. Its approximate surface tension, measured by the capillary rise method (Glasstone, 1946), was 19·5 dynes/cm.

Collection of urine. Full specimens, preserved with thymol, were collected from three patients under prolonged observation, and midstream specimens, preserved with $CHCl_3$, from all others. These were centrifuged, tested for the presence of protein (25% (w/v) salicylosulphonic acid), reducing substances (Benedict's solution) and bile salts (Hay's test) and, where necessary, made just acid with acetic acid. The surface tension of these specimens remained unchanged for 30 hr.

Procedure. Measurements were made in daylight, facing a north window. Urine was added dropwise at about 1 drop every 2 sec. to the surface between the two barriers 210–220 mm. apart, from a 1 ml. pipette delivering 0·985 ml., held at about 60° to the horizontal. Indicator oil was added 14–16 sec., later, to give a surface 25–30 mm. in diameter. During the next 4–6 sec. the surface area was reduced until the oil film was violet when observed at an angle of 45°. After a pause of 28–32 sec. the area was gradually (4–6 sec.) diminished until the oil film became a first-order sky-blue.

The distance between the inner edges of the barriers was read, by means of a transparent ruler, to the nearest mm. on both sides of the tray, and the average was noted ('tray reading'). All determinations were made in duplicate; the results usually agreed within 4 mm. up to tray readings of 100 mm. and within 8 mm. at higher values. Where the tray reading exceeded 150 mm. the urine was diluted with water and the measurement repeated. Tray readings were converted into 'apparent protein' values by reference to the standard protein curve (Fig. 1). Results were reported to the nearest 0·5 mg./100 ml. urine.

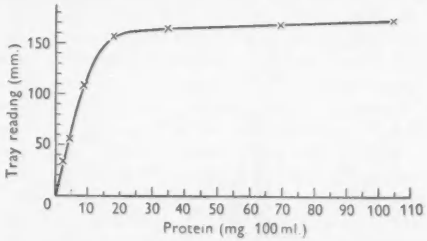

Fig. 1. Standard protein curve. Surface film measurements of diluted aqueous standard bovine albumin solutions.

Standard protein curve. A solution of bovine albumin in glass-distilled water was employed. It contained 1·49 g. N/100 ml. (Kjeldahl). Assuming the albumin to contain 15·2% N (Putnam, Erickson, Volkin & Neurath, 1943) this corresponds to 15·58 g. albumin/100 ml. solution. The carbohydrate content, determined by the method of Lustig & Nassau (1941) against a standard glucose solution, was 0·24 g./100 g. albumin. Surface-tension determinations were carried out on various aqueous dilutions of this solution. Tray readings (in mm.) are plotted directly against albumin content (mg./100 ml. urine) in Fig. 1. All apparent protein values are thus expressed in terms of bovine albumin.

Observations relating to the method. The area of the indicator drop is not strictly controlled, and variations in its size affect the total measured area. This error is minimized by the relatively small area occupied by the oil drop which was 1–2% of the total expanded surface. Where the initial

diameter of the drop diverged visibly from 25 to 30 mm. the determination was repeated.

A time factor, due partly to the ageing of the protein film (Langmuir & Waugh, 1940) and partly to the indicator oil, operates both in the expanded and the compressed state. The area at constant pressure decreases with time of standing, but the rate of decrease is slow if, as in the conditions described, reading is effected 50–60 sec. after adding the urine. Longer standing is impracticable owing to the slow disintegration of the indicator oil.

Blank determinations with the apparatus and oil, omitting urine or adding 1 ml. water instead, gave tray readings of 5 mm. or less. At these small areas there was usually some leakage across the barriers.

Gunton & Burton (1947) found a second determination after precipitating the protein unnecessary for most purposes. They refer to Tsuchiya's reagent as satisfactory, but do not give details for its use. When used according to the Shevky-Stafford method (Kolmer & Boerner, 1945) this reagent interfered considerably with the surface tension of aqueous solutions and, owing to leakage due to ethanol, failed to give stable surface areas.

RESULTS

'*Apparent protein*' *content of random samples from healthy subjects and tuberculosis cases*

The normal group of forty-two cases consisted of healthy hospital staff, twenty-six male and sixteen female, between the ages of 18 and 60 years old. The thirty-eight tuberculosis cases (twenty-five male and thirteen female) were also between 18 and 60 years old. No attempt was made to control diets or the time of collection of the urine. Some of the tuberculosis group had a mild pyrexia; the qualitative Benedict test was positive in three cases and Hay's test in one case, while tests with 25% (w/v) salicylosulphonic acid showed traces of protein in seven cases.

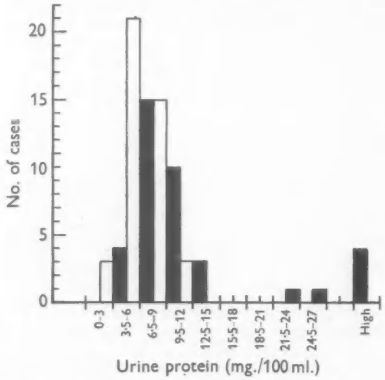

Fig. 2. Frequency distribution of apparent protein in random samples of urine from tuberculosis cases (■) and normal controls (□).

The frequency distribution of all cases of both groups is shown in Fig. 2. Four patients had an apparent proteinuria of over 27 mg./100 ml. These are not included in the calculation below, since reference to the frequency distribution chart suggests that they form a group apart. The 'apparent protein' content of the normal group ranged from 1·5 to 11·5 mg./100 ml., mean: 5·6 mg./100 ml., S.D.: 2·03. The 'apparent protein' content in the tuberculosis group was upwards of 3·5 mg./100 ml., mean of the 3·5–27 mg. group: 9·6 mg./100 ml., S.D.: 4·33.

Gunton & Burton (1947) record an average protein content of 3·7 mg./100 ml. for normal urine. The corresponding value obtained by Wang & Wu (1937) is 9 mg./100 ml.

Dilution curves

The protein content of the urine from five patients with various diseases was determined by the use of 3% (w/v) salicylosulphonic acid and comparison with permanent standards (King & Haslewood, 1936). The urines were diluted several times to give solutions of known protein contents, and surface tension curves were obtained (Fig. 3). The tray readings of these dilutions as well as of the albumin standard solution were in an approximately linear proportion to the logarithm of the protein concentration up to about 20 mg./100 ml. (Fig. 4).

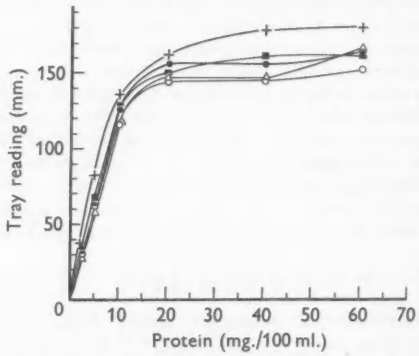

Fig. 3. Surface film measurements on human urine, using dilutions of known protein content. E. T. (pulmonary tuberculosis), +—+; N. S. (diabetes mellitus, pulmonary tuberculosis and chronic pyelonephritis), ○—○; F. W. (amyloidosis), ●—●; M. H. (diabetes mellitus and hypertensive heart failure), △—△; T. J. (coronary thrombosis), ■—■.

'*Apparent protein*' *excretion in individuals*

Since no data were available to show how the protein excretion of any individual varied throughout the day, it was not known whether a single pro-

tein determination was in any way representative of any one case. The excretion of 'apparent protein' was therefore investigated in six cases, all males.

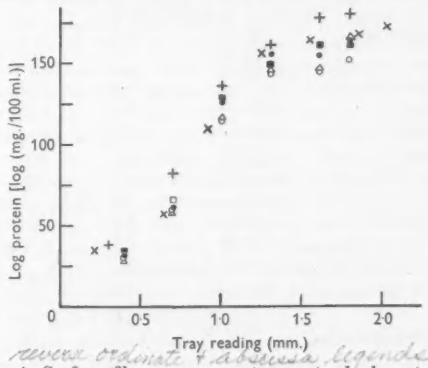

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Fig. 4. Surface film measurements on standard protein solution and on human urine, using dilutions of known protein content. (Figs. 1 and 3 redrawn.) Standard bovine albumin solution, ×; E. T., +; N. S., ○; F. W., ●; M. H., △; T. J., ■.

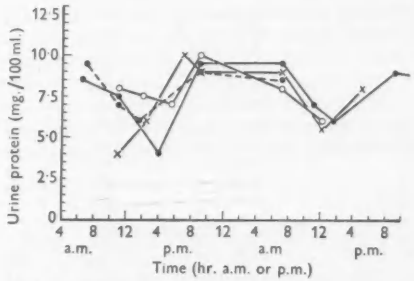

Fig. 5. 'Apparent protein' excretion in consecutive urine specimens. A. S. (pulmonary tuberculosis). Urine collection starting 15. iii. 50 (total excretion: 73 mg./24 hr.) ●—●, and ●- - -●; starting 18. iii. 50 (72 mg./24 hr.) ○—○; starting 22. iii. 50 (61 mg./24 hr.) ×—×.

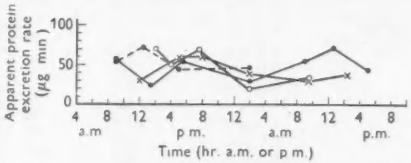

Fig. 6. A. S.: rate of excretion of 'apparent protein'. For notation see Fig. 5.

Three were healthy young adults and three patients with pulmonary tuberculosis, 30–40 years old, on bed rest but up in the afternoons. The diet was uncontrolled throughout. The normal cases collected midstream specimens of all urine passed over a period of 24 hr. or longer, and the fluctuation in 'apparent protein' content was noted. All urine from the patients was collected, and the volumes were recorded together with the 'apparent protein' contents. This made it possible to calculate the 'apparent protein excretion rate', expressed in μg. 'apparent protein' excreted per min., and referred to the time half-way between passing two successive specimens. The figures were calculated to the nearest 0·5 hr. Total daily excretions of 'apparent protein' were computed from the protein contents of complete specimens. The results of one case (A. S.) are shown in Figs. 5 and 6; all others are given in Table 1.

DISCUSSION

These determinations require accurate timing and a uniform technique. Once these have become a matter of routine, a large number of analyses can be made more simply than with Gunton & Burton's (1947) equipment. A larger trough and an indicator oil of higher surface pressure enable most determinations to be carried out without diluting the urine. Measuring the distance between two movable barriers on both sides of the tray renders the usual barrier, rigidly fixed to a guide piece and scale, unnecessary; this simpler form of apparatus is easier to clean and to coat. Except at small surface areas, paraffin-coated surfaces are sufficiently hydrophobic, and no ferric stearate need be used.

The experimental error due to variations in the size of the indicator drop is minimized by the larger surface area of the protein film. A greater gain in accuracy is the high pressure of the indicator oil. Gunton & Burton (1947) measure the area of protein layers at a surface pressure range where these are highly compressible (Adam, 1941). The present method lessens this source of error by using a range where the area of the protein film is relatively stable to minor changes in pressure. The general agreement between duplicate determinations indicates an overall accuracy no less than that recorded by Gunton & Burton.

The nature of the protein film is not clear. It is of course not a pure protein, and the method of adding the urine makes it an inhomogeneous surface layer (Hughes & Rideal, 1932). It is a solid film, as may be shown by sprinkling French chalk on the half-compressed surface. The time effect may be due to the main film constituent or to non-protein impurities. Of these, bile salts suggest themselves as being both surface active and adsorbable on proteins; a negative Hay's test is no indication that small amounts are not present. Marfori (1941) found that the addition of sodium glycocholate to normal urine had an unexpectedly small effect on the surface tension, but this work would need extending before its conclusions could be applied here. The different physical

Table 1. *Diurnal variation in urinary 'apparent protein' excretion*

(Dates refer to the collection of the first specimen in each series. Figures in brackets are 'apparent protein' excretions in mg./24 hr.)

Date	Time of collection	Protein (mg./100 ml.)	Excretion rate (μg./min)
	Subject, A.T., normal		
3. xi. 49	11.30 a.m.	9·5	—
	2.0 p.m.	11·5	—
	6.0 p.m.	8·0	—
	9.0 p.m.	7·5	—
	7.30 a.m.	5·5	—
	Noon	9·0	—
20. xi. 49	11.0 p.m.	5·5	—
	8.0 a.m.	5·0	—
	11.0 a.m.	10·0	—
	4.30 p.m.	6·5	—
	7.0 p.m.	5·5	—
	11.0 p.m.	6·5	—
	8.0 a.m.	7·0	—
28. xi. 49	11.0 p.m.	6·5	—
	8.30 a.m.	4·5	—
	1.0 p.m.	10·5	—
	6.0 p.m.	8·5	—
	9.0 p.m.	7·0	—
	9.0 a.m.	3·0	—
2. xii. 49	8.30 p.m.	5·5	—
	10.30 p.m.	4·5	—
	8.30 a.m.	3·5	—
	1.30 p.m.	10·0	—
	5.0 p.m.	7·0	—
	8.0 p.m.	4·0	—
	Subject, J.W., normal		
18. xii. 49	11.30 p.m.	4·0	—
	8.30 a.m.	11·5	—
	11.0 a.m.	4·0	—
	3.0 p.m.	4·0	—
	7.0 p m.	4·0	—
	Midnight	7·0	—
	8.0 a.m.	5·5	—
20. xii. 49	Midnight	4·5	—
	8.0 a.m.	3·0	—
	1.0 p.m.	2·5	—
	6.0 p.m.	4·0	—
	11.0 p.m.	2·5	—
	8.0 a.m.	42·0	—
8. i. 50	Midnight	2·5	—
	8.30 a.m.	8·5	—
	11.30 a.m.	3·5	—
	5.0 p.m.	8·5	—
	8.0 p.m.	5·5	—
	Midnight	4·5	—
11. i. 50	11.0 p.m.	7·5	—
	8.0 a.m.	7·5	—
	11.30 a.m.	2·5	—
	6.0 p.m.	5·5	—
	11.0 p.m.	5·0	—
	0.30 a.m.	3·0	—
	8.0 a.m.	5·0	—
	Subject, L.S., normal		
27. ii. 50	10.30 p.m.	6·0	—
	7.0 a.m.	6·0	—
	11.30 a.m.	7·0	—
	2.30 p.m.	7·0	—
	7.0 p.m.	5·5	—
	10.30 p.m.	5·0	—
8. iii. 50	1.30 p.m.	5·0	—
	7.30 p.m.	6·0	—
	7.30 a.m.	6·0	—
	11.0 a.m.	7·0	—
	2.30 p.m.	5·0	—
	6.30 p.m.	4·5	—
21. iii. 50	1.30 p.m.	7·0	—
	7.30 p.m.	7·0	—
	11.0 p.m.	8·5	—
	8.0 a.m.	8·5	—
	2.30 p.m.	8·0	—
29. iii. 50	12.30 p.m.	7·0	—
	2.0 p.m.	7·0	—
	5.30 p.m.	6·5	—
	7.30 p.m.	8·0	—
	11.0 p.m.	9·5	—
	8.0 a.m.	6·0	—
	1.0 p.m.	8·0	—
	Subject, H.R., tuberculosis		
6. xii. 49 (68)	6.30 a.m.	2·0	80
	9.30 a.m.	8·5	48
	1.30 p.m.	6·0	50
	7·0 p.m.	8·5	148
	9.0 p.m.	8·5	18
	6.30 a.m.	5·5	
9. xii. 49 (69)	2.0 p.m.	6·5	59
	5.0 p.m.	5·5	70
	9.0 p.m.	8·0	23
	7.0 a.m.	6·5	66
	10.30 a.m.	6·0	
12. xii. 49 (71)	7.0 a.m.	6·5	76
	10.0 a.m.	6·5	50
	1.30 p.m.	5·0	44
	5.0 p.m.	4·0	104
	9.0 p.m.	12·5	25
	6.30 a.m.	6·0	
15. xii. 49 (58)	10.0 a.m.	5·0	78
	1.30 p.m.	8·0	34
	5.0 p.m.	3·0	23
	9.30 p.m.	3·0	33
	7.0 a.m.	9·0	
	Subject, F.B., tuberculosis		
22. iii. 50 (62)	11.30 a.m.	11·5	59
	1.30 p.m.	7·5	42
	4.0 p.m.	5·5	83
	6.0 p.m.	10·0	64
	7.30 p.m.	10·5	52
	9.0 p.m.	9·5	19
	6.0 a.m.	9·5	57
	9.0 a.m.	8·5	60
	11.0 a.m.	8·5	60
	1.0 p.m.	8·0	
25. iii. 50 (63)	5.30 p.m.	7·5	90
	7.0 p.m.	13·5	56
	9.0 p.m.	9·0	16
	6.0 a.m.	9·5	42
	10.0 a.m.	12·5	56
	1.0 p.m.	10·0	87
	2.0 p.m.	4·5	58
	6.0 p.m.	8·0	286
	6.30 p.m.	9·0	
29. iii. 50 (51)	4.30 p.m.	6·0	54
	7.0 p.m.	9·0	60
	9.0 p.m.	11·0	18
	6.30 a.m.	7·0	32
	9.30 a.m.	9·0	79
	11.0 a.m.	9·5	39
	1.30 p.m.	6·5	33
	4.0 p.m.	5·0	50
	6.0 p.m.	6·0	127
	6.30 p.m.	8·5	

nature of the films obtained by this technique and by Gunton & Burton's method is shown well by the shape of the two standard protein curves.

The urinary proteins from cases with different diseases show no clear variation (Fig. 3), and all agree fairly closely with the standard protein curve. Most of the indicator oil films in this investigation were 'smooth circular' in the expanded state (Schaefer, 1938), but one tuberculosis urine gave a circular film surrounded by a halo-like zone, and one showed a 'star-like' expansion pattern. The standard protein solution and all urines used for the dilution curves of Fig. 3 gave 'star-like' oil drops at high protein concentrations, the points of the stars being toned down considerably below concentrations of 20 mg./100 ml. The shape of the oil film shows no qualitative difference in protein structure for the normal and tuberculous groups.

The study of the diurnal variation in the 'apparent protein' output of six cases allows one definite conclusion to be made: that while the collection of random samples can be a satisfactory way of comparing groups, no single specimen of urine is sufficiently representative of the average daily output of protein in the urine of any one person. Other observations are only of a tentative nature. Individual cases show some regularity both in the 'apparent protein' concentration of urine and its rate of excretion. Variations of the two quantities are related but not parallel, and their comparison in a greater number of cases may be of value in a study of postural proteinuria, of which these cases seem to show examples, and clinical conditions associated with low protein excretion. The day-to-day variation in 24 hr. protein excretion also seems more constant in any one person than has been reported by Wang & Wu (1937).

SUMMARY

1. The surface-tension method of Gunton & Burton (1947) has been simplified and modified for use with undiluted urine. Possible increases in the experimental error are offset by increased accuracy in the final step, where an indicator oil of a higher surface tension is used to measure the area at constant pressure of a solid surface film.

2. This method has been used to determine the 'apparent protein' content of urine from healthy and tuberculous subjects, the latter group giving higher values.

3. The fluctuation in the apparent proteinuria at various times of the day has been followed in six cases, and some regularity has been noted.

I should like to express my thanks to Dr E. Nassau (Pathologist, Harefield Hospital), at whose suggestion this work was carried out, for continuous help, discussions and suggestions; to Mr E. A. Evans (Chief Chemist, Research Department, C. C. Wakefield Ltd., Hayes) for a valuable discussion and a gift of the Technical White Oil used as indicator; and to many patients and members of the medical, laboratory and nursing staff of St Andrew's Hospital, London, E. 3, for organizing the collection of specimens, and their help throughout the investigation.

REFERENCES

Adam, N. K. (1941). *The Physics and Chemistry of Surfaces*, 3rd ed. Oxford: University Press.

Glasstone, S. (1946). *The Elements of Physical Chemistry*, New York: D. Van Nostrand.

Gunton, R. & Burton, A. C. (1947). *J. clin. Invest.* **26**, 892.

Hughes, A. H. & Rideal, E. K. (1932). *Proc. roy. Soc.* A, **137**, 70.

King, E. J. & Haslewood, G. A. D. (1936). *Lancet*, **2**, 1153.

Kolmer, J. A. & Boerner, F. (1945). *Approved Laboratory Technic*, 4th ed. New York: D. Appleton-Century Co.

Langmuir, I. & Waugh, D. F. (1940). *J. Amer. chem. Soc.* **62**, 2771.

Lustig, B. & Nassau, E. (1941). *Amer. Rev. Tuberc.* **43**, 817.

Marfori, L. (1941). *Policlinico*, **48**, 323. Taken from *Chem. Abstr.* 1947, **41**, 4213g.

Putnam, F. W., Erickson, J. O., Volkin, E. & Neurath, H. (1943). *J. gen. Physiol.* **26**, 513.

Schaefer, V. J. (1938). *J. phys. Chem.* **42**, 1089.

Wang, C.-F. & Wu, H. (1937). *Chin. J. Physiol.* **12**, 37.

A Glucuronide-Decomposing Enzyme from Rumen Micro-organisms

1. PREPARATION AND ASSAY

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The most characteristic feature of ruminant digestion lies in the use of cellulose and the associated ill-defined polysaccharides which are collectively known as hemicellulose. Digestion of these substances is brought about by the mixed microbial population of the rumen, and considerable study has been made of the end products of this process, in particular of the volatile fatty acids which are absorbed through the rumen wall into the blood stream of the host (Elsden & Phillipson, 1948). Such studies, however, cast little light on the decomposition of cellulose or hemicellulose in its early stages, since qualitatively similar end products result from the digestion of starch and simple sugars in the rumen. The scattered and inconclusive evidence available from studies of cellulose degradation by bacterial cultures (for reviews see Norman & Fuller, 1942; Fåhræus, 1947; Hungate, 1950) suggests that glucose and cellobiose may be intermediates in this process. Caution is, however, necessary in attempting to apply conclusions arrived at with individual organisms to changes brought about by a complex population such as that of the rumen (Norman & Fuller, 1942; Elsden & Phillipson, 1948). If reducing sugars are formed in the rumen from cellulose or hemicellulose, it would appear that the insolubility of the substrate makes the process a slow one, followed by rapid destruction of the sugars by the micro-organisms. Such considerations make a direct approach to the study of the enzymic mechanism of cellulose and hemicellulose fission in the rumen a matter of very great difficulty. It has been suggested (e.g. Boswell, 1941), although on evidence which is open to criticism (Fåhræus, 1947), that microbial fission of the cellulose chain involves preliminary oxidation to a polyglucuronide. Be that as it may, glucuronic acid residues enter into the composition of certain hemicellulose polysaccharides in the plant (Norman, 1937).

It was considered that the identification and characterization of β-glycosidases present in the total rumen contents in significant amounts would constitute a useful contribution to knowledge of the special features of ruminant digestion. For the initial identification of such enzymes, it was proposed to make use of soluble β-glycosides containing aglycones which, in the free state, are stable and readily detected in the presence of intact rumen micro-organisms. This approach permits treatment of the rumen liquor as though it were one tissue.

Biosynthetic phenolphthalein β-D-glucuronide, which has been recommended for use in the assay of animal β-glucuronidase by Talalay, Fishman & Huggins (1946), appeared to be a suitable substrate. As described below, a fraction of the micro-organisms in the sheep rumen has been found to contain an enzyme or system of enzymes which liberates phenolphthalein from the glucuronide. The present paper deals with the preparation of the enzyme in cell-free extract and the determination of optimum conditions for assay.

EXPERIMENTAL AND RESULTS

General methods

Enzyme assay. On the basis of results obtained in early experiments, the following general procedure was adopted. To 3 ml. 0·1 M-phosphate buffer, pH 6·1, were added 0·5 ml. 0·004 M-phenolphthalein glucuronide solution (adjusted to pH 6·1) and 0·5 ml. enzyme preparation (rumen liquor, cell suspension or cell-free extract). After incubating the mixture at 37° for 1 hr., the reaction was arrested and the colour of the free phenolphthalein developed by addition of 4 ml. of a buffer solution, pH 10·7 (0·4 M-glycine, 0·2 M-NaCl and 0·25 M-Na_2CO_3). The mixture was centrifuged at 1500 *g* for 15 min., and the intensity of the red colour in the supernatant was read with a Spekker photoelectric absorptiometer, using Ilford no. 605 yellow-green filter. Results were read from a calibration curve constructed by replacing substrate and enzyme in the mixture by a phenolphthalein solution containing 10–80 μg., and are expressed as μg. phenolphthalein liberated in 1 hr. at 37°.

The addition of Na_2CO_3 was found to increase the efficiency of the glycine buffer recommended by Talalay *et al.* (1946) for colour development. Increasing the final Na_2CO_3 concentration beyond 0·125 M, however, led to rapid colour fading.

When phenolphthalein was added to suspensions of rumen micro-organisms and incubated as described above, the recovery was quantitative.

Nitrogen determination. Kjeldahl digestion was carried out according to the procedure of Chibnall, Rees & Williams (1943). NH_3 was evolved in the Markham still (Markham, 1942), absorbed in 2% boric acid containing methyl red and bromocresol green (see Conway, 1947), and titrated with 0·01 N-HCl.

Measurement of pH was done with the glass electrode.

Table 1. *Fractionation of rumen contents*

Fraction no.	Relative centrifugal force (*g*)	Period of centrifuging (min.)	Enzyme activity (μg. phenolphthalein) per mg. total N	Enzyme activity (μg. phenolphthalein) Total in fraction	Microscopic observations
1	15	2	80	6,000	Large protozoa, food debris and a few organisms from 2
2	1500	30	240	21,000	Quin's organism, small protozoa and large bacteria
3	5000	30	60	800	Small bacteria and a few organisms from 2
4	Supernatant from 3		0	0	Almost cell-free

Rumen liquor. Cheviot sheep with permanent rumen fistulae were fed daily with 1 lb. hay at 8 a.m., 0·5 lb. concentrate (2 parts linseed oil cake and 1 part crushed oats) at 11 a.m. and 1 lb. hay at 8 p.m. The animals had free access to water and salt lick. Rumen liquor was withdrawn at 10.30 a.m. and strained through eight layers of surgical gauze. Eight animals have been used in the course of this work. Under the conditions stated, no marked variation in the composition of the rumen microbial population was observed from animal to animal or from day to day. The pH was 6·0–6·2.

Centrifugal fractionation of rumen contents

On the basis of preliminary experiments in which the strained rumen liquor was centrifuged at different speeds for varying times, the procedure shown in Table 1 was drawn up for fractionating the micro-organisms according to their enzyme activity. The precipitate obtained at each stage was resuspended in water and samples were taken for measurement of enzyme activity and total N. Whilst no systematic attempt was made to assess the relative importance of the different rumen micro-organisms in this connexion, Table 1 suggests that the enzyme activity was associated with the medium-sized species which sediment in fraction 2. These comprise Quin's yeast-like organism (Quin, 1943), small protozoa and large bacteria. The activities of fractions 1 and 3, consisting mainly of large protozoa and small bacteria respectively, were probably due to contamination with fraction 2. Large protozoa (*Holotrich* and *Oligotrich* ciliates) in bacteria-free preparations, and enriched cultures of bacteria fermenting xylose and glucose (small cocci and cocco-bacilli), obtained from the sheep rumen by Dr A. E. Oxford in this Institute, were unable to decompose phenolphthalein glucuronide. The fact that the final supernatant in Table 1 had no activity shows that the enzyme was neither extracellular nor derived from body fluids.

Preparation of the enzyme

Stage A. Fraction 2 of the rumen micro-organisms (see Table 1) was washed on the centrifuge four times with thrice its volume of water to remove pigmented material, and resuspended in water.

Stage B. To obtain a cell-free extract, the suspension was shaken in small batches with half volumes of Ballotini, Grade 12 (Chance Bros., Ltd.) in the Mickle Tissue Disintegrator (Mickle, 1948) for 1·5 hr. The aqueous layer was centrifuged at 8300 *g* for 30 min. and the supernatant separated. Precipitated material was suspended in water and put through the extraction procedure once more. The combined extracts were again centrifuged at 8300 *g* for 30 min., to give a supernatant which was almost free from microscopic particles.

Stage C. Before proceeding with $(NH_4)_2SO_4$ fractionation, it was found necessary to remove mucilaginous material. Since the enzyme is unstable at acid pH (see below), the following procedure was adopted. To the cell-free solution was added M-acetate buffer, pH 4·3, till the pH of the mixture was 4·5. After centrifuging in chilled cups at 1500 *g* for 15 min., the supernatant was separated and brought to pH 6·1 with 2 N-NaOH.

Stage D. To the enzyme solution was added one-tenth of its volume of M-phosphate buffer, pH 6·1, followed by sufficient saturated $(NH_4)_2SO_4$ solution to give a final saturation of 30%. The bulky precipitate obtained on centrifuging at 1500 *g* for 15 min. was discarded. The supernatant was brought to 70% saturation with $(NH_4)_2SO_4$ and again centrifuged. The supernatant was discarded, and the precipitate dissolved in water and dialysed overnight against running tap water. Attempts to narrow the limits for $(NH_4)_2SO_4$ fractionation led to considerable loss of enzyme.

Table 2 shows the recoveries obtained at various stages in the above procedure.

Table 2. *Recovery of enzyme at various stages of preparation*

Stage	Vol. (ml.)	Total enzyme activity (μg. phenolphthalein)
A, washed micro-organisms	250	51,000
B, cell-free extract	350	29,300
C, solution after adjusting *B* to pH 4·5	400	21,500
D, ppt. at 30–70% saturation with $(NH_4)_2SO_4$	150	15,000

Tests for the existence of a coenzyme

No suggestion was obtained in the experiments described above of the existence of a coenzyme. Nevertheless, experiments were carried out to test this point directly. Dialysis of the cell-free extract at stage *B* for 20 hr. against running tap water caused no significant fall in enzyme activity. Immersion of the enzyme preparation in boiling water for 3 min. caused complete loss of activity. This preparation when added to an unboiled enzyme extract produced no increase in the activity of the latter.

Variation in activity with pH

Fig. 1 shows the effect of varying the pH on the rate of decomposition of phenolphthalein glucuronide by rumen preparations. To 3 ml. phosphate-citrate buffer (mixtures of 0·2 M-Na_2HPO_4 and 0·1 M-citric acid) were added 0·5 ml. enzyme preparation (stage *B*) and 0·05 ml. 0·01 M-phenolphthalein glucuronide solution. The mixture was incubated at 37° for 1 hr. There was little change in the pH during incubation. After addition of 4 ml. Na_2CO_3-glycine buffer, the contents of all tubes were brought to pH 10·3–10·5 with NaOH and diluted to 10 ml. As can be seen from Fig. 1, the activity showed a sharp optimum at pH 6·1.

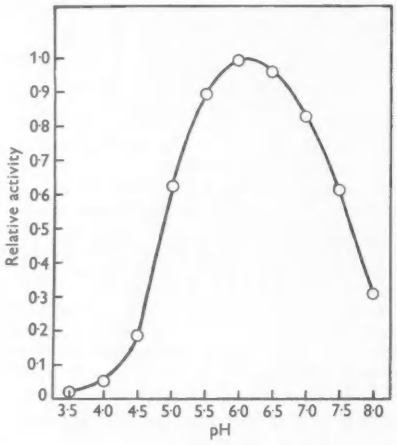

Fig. 1. Effect of pH on the rate of decomposition of phenolphthalein glucuronide by rumen enzyme in phosphate-citrate buffer.

The activity of an enzyme preparation at pH 6·1 was the same in phosphate-citrate, phosphate, citrate or acetate buffer.

Variation in stability with pH

The stability of the enzyme at varying pH is illustrated in Fig. 2. About 15 ml. enzyme preparation (stage *B*) adjusted to the required pH with HCl or NaOH, was incubated at 37° for 15 min. or 1 hr. The preparation was then rapidly cooled to room temperature and brought to pH 6·1, and the activity was assayed in the usual way. Comparison of Figs. 1 and 2 makes it evident that the peak in the pH-activity curve (Fig. 1) was largely determined by irreversible inactivation of the enzyme outside the pH range 6–7.

Variation in activity with substrate concentration

The effect of varying the substrate concentration on the rate of decomposition of phenolphthalein glucuronide by a rumen enzyme preparation (stage *D*) in phosphate buffer, pH 6·1, is shown in Fig. 3. Similar relationships were found in other experiments. It can be seen that inhibition by excess substrate was pronounced. The experimental results were analysed by the graphical method of Lineweaver & Burk (1934), as shown in Fig. 4. A value of $3·05 \times 10^{-5}$ M was obtained for K_s, the dissociation constant of the active enzyme-substrate complex, whilst n, the number of substrate molecules

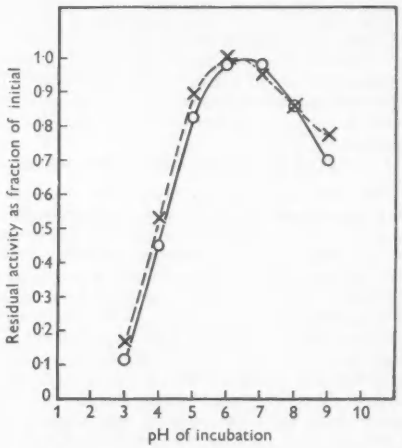

Fig. 2. Inactivation of the enzyme after 15 min. (× - - ×) and after 1 hr. (○ — ○) at 37° and varying pH. Final assay at pH 6·1. Results expressed as fraction of the activity before incubation.

per active enzyme centre in the inactive complex was 2 (the actual value given by Fig. 4 is 1·97), and K_2, the dissociation constant of the inactive complex, was 0·0126 M. The optimum activity of the enzyme was reached at a substrate concentration of $6·2 \times 10^{-4}$ M ($=\sqrt{[K_s K_2]}$, cf. Lineweaver & Burk, 1934). A substrate concentration of 0·0005 M was selected for routine enzyme assay.

Fig. 4 also gives a value of 38·2 μg. phenolphthalein liberated for V_m, the theoretical maximum velocity of reaction in the absence of inhibition for the particular enzyme preparation employed. This contrasts with a figure of 34·8 μg. phenolphthalein for the observed optimum velocity of hydrolysis by the preparation. The line shown in Fig. 3 was drawn

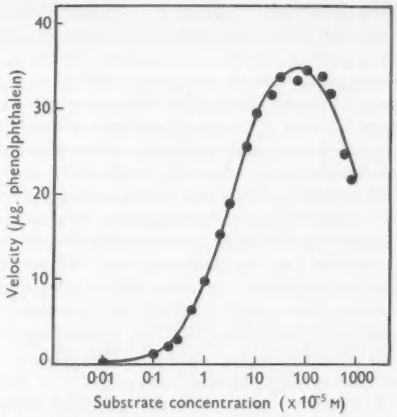

Fig. 3. The effect of varying the substrate concentration (S) on the rate of decomposition (v) of phenolphthalein glucuronide. The points were obtained experimentally whilst the line was drawn through values calculated by substituting for K_S, K_2, n and V_m (see Fig. 4) in the equation

$$v = \frac{V_m S}{S + K_S + S^n/K_2}.$$

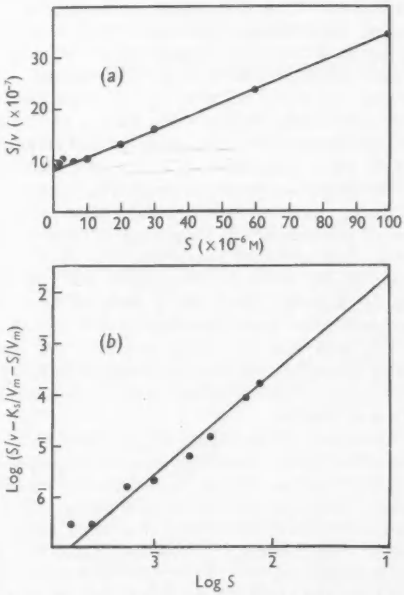

Fig. 4. Derivation of K_S, K_2, n and V_m by the method of Lineweaver & Burk (1934). (a) When S/v is plotted against S at low substrate concentrations, the slope is $1/V_m$ and the intercept at $S=0$ is K_S/V_m. (b) When $\log(S/v - K_S/V_m - S/V_m)$ is plotted against $\log S$ at high substrate concentrations, the slope is n and the intercept at $S=1$ is $-\log K_2 V_m$.

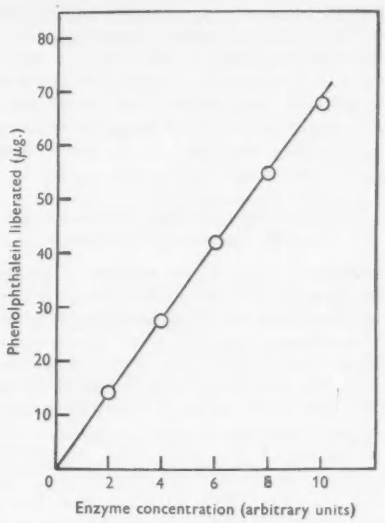

Fig. 5. Phenolphthalein liberated by varying enzyme concentrations under standard conditions.

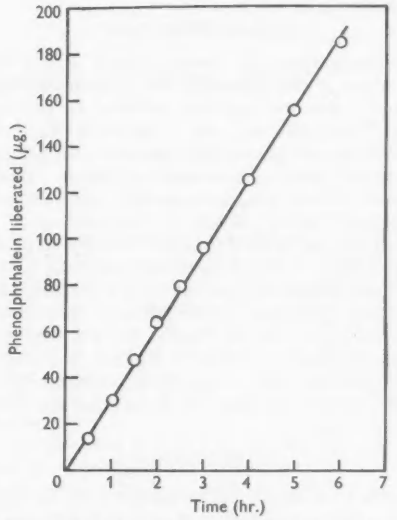

Fig. 6. Phenolphthalein liberated after varying periods of incubation.

through values of v calculated by substituting for K_S, K_2, n and V_m in the equation

$$v = \frac{V_m S}{S + K_S + S^n/K_2},$$

where v is the reaction velocity at different values of S, the substrate concentration. There was excellent

agreement between the experimentally derived points and the line given by the equation.

While the mechanism of this reaction remains a matter of conjecture the simplicity of the kinetics suggests that they are derived from a single reaction, either a simple hydrolysis of the glucuronide or the rate-determining reaction in a more complex process.

Effect of varying the enzyme concentration and the duration of hydrolysis

As shown in Fig. 5, the amount of phenolphthalein liberated in 1 hr. at 37° and pH 6·1 (phosphate buffer) from 0·0005M-phenolphthalein glucuronide was directly proportional to the concentration of rumen enzyme (stage *D*). Experiments carried out under the same conditions, but in which the incubation period was varied and the enzyme concentration kept constant, showed the reaction velocity to be unchanged after 6 hr. (Fig. 6). Since the amounts of phenolphthalein liberated after incubation for more than 2 hr. were too great for direct reading with the Spekker absorptiometer, suitable samples of the reaction mixture were diluted to 4 ml. with phosphate buffer prior to colour development.

Action of saccharic acid

Karunairatnam & Levvy (1949) found that hydrolysis of phenylglucuronide by preparations of animal β-glucuronidase was inhibited by saccharic acid. The inhibition was 50% with 2×10^{-4}M-saccharate, and practically complete with less than 0·01M (substrate concentration 0·015M). Hydrolysis of 0·00125M-phenolphthalein glucuronide in phosphate buffer, pH 6·1, by preparations of the rumen enzyme (stage *D*) showed only 8% inhibition in presence of 0·01M-saccharate and 44% with 0·1M-saccharate. With mouse-liver β-glucuronidase, however, hydrolysis of 0·00125M-phenolphthalein glucuronide in acetate buffer, pH 4·5, was completely inhibited by 0·01M-saccharate. It is evident that saccharic acid causes little inhibition of the rumen enzyme compared with its effect on animal glucuronidase.

DISCUSSION

Classification of this rumen glucuronide-decomposing enzyme awaits studies of its specificity and of its possible action on polysaccharides in the diet of the sheep. These in turn require preparation of the enzyme in a comparatively pure state. It must also be shown that the sole reaction product from phenolphthalein glucuronide, apart from free phenolphthalein, is glucuronic acid. It should be borne in mind that the liberation of phenolphthalein may result, not from simple hydrolysis of the glucosidic link, but from the combined actions of a decarboxylase and xylosidase, or some other such complex process. Whilst direct investigation of this point is contemplated, the simplicity of the kinetic data suggests that in the present instance we are dealing with a hexuronidase. Moreover, the loss in enzyme activity in passing from the whole cell suspension to the partially purified cell-free extract was smaller than might be expected for a complex system involving steps such as decarboxylation.

Since the source of this enzyme is not a pure culture, but a mixed suspension of micro-organisms, it is possible that the final activity is influenced by factors contributed by more than one type of organism. This state of affairs is, however, that obtaining in the rumen, and the present approach avoids the difficulties of interpretation which attend pure culture work in this field.

It is considered possible that the natural substrate for this enzyme is not a conjugated glucuronide such as is found in urine, but a plant polysaccharide containing uronic acid residues, or a fission product thereof. It is interesting to note that preliminary observations have been made in this Institute (Moir & Marsh, 1950) in which variations in the number of Quin's organisms (Quin, 1943) present in rumen liquor *in vivo* appeared to be correlated with variations in the activity of the glucuronide-decomposing enzyme. No evidence, however, exists to suggest that this micro-organism is particularly concerned in the early stages of hemicellulose degradation.

Whilst enriched cultures of xylose-fermenting bacteria from the rumen were unable to liberate phenolphthalein from its glucuronide, our colleagues Dr A. E. Oxford and Mr C. A. Marsh have found that they develop this property after growth on a medium containing menthyl glucuronide. The adaptive response to the presence of a conjugated glucuronide appears analogous to that shown by other micro-organisms, for example *Escherichia coli* (Buehler, Katzman & Doisy, 1949). Since there is no evidence to suggest that small bacteria of the xylose-fermenting type with the ability to decompose glucuronides normally occur in the rumen, it is not proposed to study the adaptive enzyme in question any further at present.

Consideration of the composition of skeletal polysaccharides in the plant suggests other compounds worthy of use as 'model' substrates in studying digestive enzymes in the rumen along the lines described in the Introduction. Use of one of these, phenyl β-D-glucoside, is contemplated in the immediate future.

SUMMARY

1. An intracellular glucuronide-decomposing enzyme has been prepared in cell-free state from medium-sized micro-organisms in the rumen of the sheep.

2. The optimum pH for the decomposition of phenolphthalein glucuronide by the enzyme was 6·1.

3. Irreversible inactivation of the enzyme occurred on incubation for a short period at a pH lower than 6 or higher than 7.

4. On the assumption that the rate of liberation of phenolphthalein from the glucuronide was governed by a single reaction a value of $3{\cdot}05 \times 10^{-5}$ M was obtained for the dissociation constant of the active enzyme-substrate complex. Excess substrate caused pronounced inhibition of the enzyme. The inactive complex contained two substrate molecules per active enzyme centre, and the second dissociation constant was 0·0126 M.

5. Saccharate caused only slight inhibition of the enzyme in comparison with its effect on animal β-glucuronidase.

The authors wish to express their gratitude to Dr A. E Oxford for assistance and advice in microbiological aspects of this work, and to Dr A. T. Phillipson for the supply of sheep with rumen fistulae.

REFERENCES

Boswell, J. G. (1941). *New Phytol.* **40**, 20.
Buehler, H. J., Katzman, P. A. & Doisy, E. A. (1949). *Fed. Proc.* **8**, 189.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Conway, E. J. (1947). *Micro-Diffusion Analysis and Volumetric Error*, 2nd ed. London: Crosby Lockwood.
Elsden, S. R. & Phillipson, A. T. (1948). *Ann. Rev. Biochem.* **17**, 705.
Fåhræus, G. (1947). *Symb. bot. upsaliens.* **9**, no. 2.
Hungate, R. E. (1950). *Bact. Rev.* **14**, 1.
Karunairatnam, M. C. & Levvy, G. A. (1949). *Biochem. J.* **44**, 599.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Mickle, H. (1948). *J. R. micr. Soc.* **68**, 10.
Moir, R. J. & Marsh, C. A. (1950). Unpublished observations.
Norman, A. G. (1937). *The Biochemistry of Cellulose, the Polyuronides, Lignin etc.* Oxford: University Press.
Norman, A. G. & Fuller, W. H. (1942). *Advanc. Enzymol.* **2**, 239.
Quin, J. I. (1943). *Onderstepoort J. vet. Sci.* **18**, 91.
Talalay, P., Fishman, W. H. & Huggins, C. (1946). *J. biol. Chem.* **166**, 757.

The Chromatographic Fractionation of Ribonuclease

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During a study of the partial hydrolysis of ribonuclease (Porter, 1950), it became necessary to separate enzymically active products from the original enzyme and other substances. In some cases the degradation appeared to be slight and little change in solubility or other physical properties seemed to have occurred. Conventional methods of protein purification, such as precipitation with salts or organic solvents proved unsuccessful, and attention was therefore turned to partition between two liquid phases. The system ammonium sulphate-water-ethanol was used very successfully by Herbert & Pinsent (1948) in the purification of bacterial catalase. In some preliminary experiments with haemoglobin (Martin, 1949), the system ammonium sulphate-water-ethylene glycol monoethyl ether ('cellosolve') was found to be less likely than most to cause denaturation. This system was therefore chosen for investigation, though ribonuclease is so stable a protein that many systems may well be satisfactory. In one case, however, the ratio of the partition coefficient of ribonuclease to that of the aggregate of its hydrolysis products was about 3:2. This system could yield a satisfactory separation only with the aid of a counter-current extraction train of very high efficiency, or of a partition chromatogram. The latter was chosen, using kieselguhr to support the stationary phase, since cellulose, starch or precipitated silica would have probably too fine a structure to admit a protein molecule.

These columns proved very successful, and revealed the presence of two enzymically active components in the original crystalline ribonuclease.

MATERIALS

Ribonuclease was prepared from ox pancreas by the method of Kunitz (1940) and also by the modified procedure of McDonald (1948). It was recrystallized several times and was homogeneous in the Tiselius electrophoresis apparatus at pH 7·4 and 8·5.

The kieselguhr was Hyflo Supercel obtained from the Johns Manville Co. Ltd., London. It was used without pretreatment.

METHODS

Ribonuclease assay was carried out as described by Kunitz (1940). The activity of the enzyme preparations is expressed in the nuclease units (N.U.) of Kunitz. One N.U. is the activity which gives rise under standard conditions to the formation of 1×10^{-3} mg. of soluble phosphorus per ml. of digestion mixture in a range of concentrations of enzymes where the amount of soluble phosphorus formed is proportional to the concentration of enzyme used.

The relative protein concentration of the concentration of the eluates was followed by measuring the absorption at 275 mμ. in a Beckman photoelectric absorptiometer.

Choice of solvent mixture and preparation of the chromatogram

The phase diagram of the system $(NH_4)_2SO_4$-water-cellosolve (Fig. 1) was determined by titrating a saturated solution of $(NH_4)_2SO_4$ in water with cellosolve until a faint opalescence showed the presence of two phases. Water was then added and the titration continued. The tie lines were determined from the $(NH_4)_2SO_4$ content of both phases of a number of arbitrary mixtures.

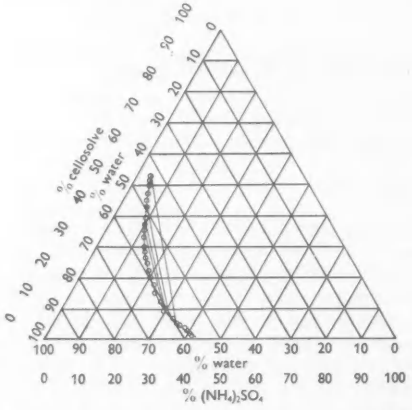

Fig. 1. Phase diagram of the system ammonium sulphate-water-ethylene glycol monoethyl ether at 20°.

Using the phase diagram a number of mixtures were chosen and tested. The choice was restricted by the limited solubility of the enzyme in aqueous phases having a high salt content, since the assay was disturbed by excess of $(NH_4)_2SO_4$ and the sample had to be diluted considerably before test. Three mixtures used successfully had the following composition (by weight):

(*a*) Water, 47%; $(NH_4)_2SO_4$, 16·5%; cellosolve 36·5%.
(*b*) Water, 55%; $(NH_4)_2SO_4$, 15%; cellosolve, 30%.
(*c*) Water, 56%; $(NH_4)_2SO_4$, 20%; cellosolve, 24%.

Of these mixtures (*c*) was perhaps the most useful and has been employed in much of this work.

The partition coefficients of the crystalline enzyme in two of the mixtures were (*a*) 10, (*b*) 6 in favour of the upper (cellosolve) layer. The organic phase had, therefore, to be the stationary one, and the aqueous the mobile phase. For reasons not fully understood the kieselguhr was wetted preferentially by the organic phase, and it was therefore unnecessary to use silane-treated silica (Howard & Martin, 1950). Indeed it has not as yet proved possible to produce columns with the aqueous phase stationary.

The column is prepared by mixing the kieselguhr with half its weight of the organic phase, making this into a slurry with excess of the aqueous phase and pouring into the chromatogram tube. For most of the work columns containing 6 g. of kieselguhr in a tube 1·2 cm. internal diameter were used. Such a column can conveniently handle 1–5 mg. ribonuclease dissolved in 2 ml. of aqueous phase.

RESULTS

The elution of the enzyme was followed both by estimating the absorption at 275 mμ. and by estimating the activity. In Fig. 2 it can be seen that these follow each other closely, but that two distinct peaks are visible instead of the single peak expected.

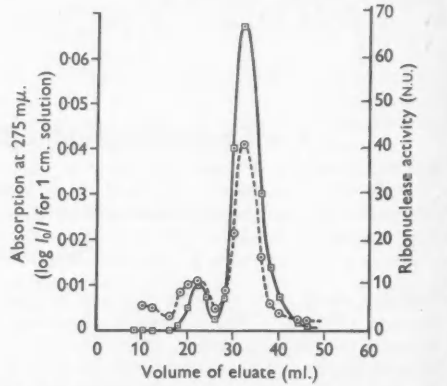

Fig. 2. Chromatogram of ribonuclease. System: 20 g. $(NH_4)_2SO_4$, 24 g. cellosolve, 56 g. water; 6 g. silica column. ——, enzymic activity; - - - -, absorption at 275 mμ.

In Fig. 3 are shown the elution curves of the same enzymic material using different solvent mixtures. It will be observed that the relative proportion of the two components as judged from the area beneath the peaks remains approximately constant, although the degree of separation varies considerably.

As it therefore appeared that the enzyme existed in two forms it was necessary to decide whether either could be an artifact of the column or of the method of preparation of the enzyme from the pancreas. Material concentrated from the eluate containing either peak could be re-run upon the chromatogram to give a clean single peak, showing that no alteration was occurring here.

In the preparation there are two apparent ways in which degradation might occur. First, in McDonald's modification of the Kunitz method of preparation, proteolytic activity, always present as contaminant in the older method, is destroyed by heating at 100° for 10 min. It was possible that some hydrolysis of

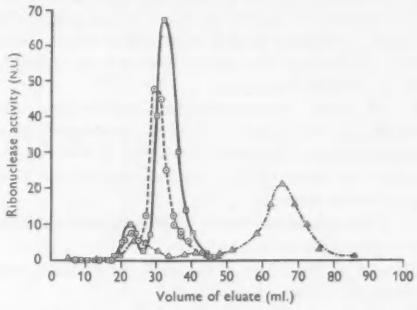

Fig. 3. Chromatograms of ribonuclease. ⊙---⊙, system: 15 g. $(NH_4)_2SO_4$, 30 g. cellosolve, 55 g. water; 6 g. silica column; ⊡—⊡, system: 20 g. $(NH_4)_2SO_4$, 24 g. cellosolve, 56 g. water; 6 g. silica column; △-·-·-△, system: 16·5 g. $(NH_4)_2SO_4$, 36·5 g. cellosolve, 47 g. water; 3 g. silica column.

amide groups or some other type of degradation might occur. Ribonuclease was therefore prepared omitting this step; nevertheless, two peaks were still present in the same ratio. As further confirmation, the main peak was collected from a chromatogram, heated to 100° for 15 min. and again run. Once more only a single peak appeared and in the same position as before heating.

The other probable explanation of the appearance of the second peak was that the powerful proteolytic enzymes of the pancreas caused some hydrolysis of the ribonuclease during the initial extraction. The method of preparation is a series of ammonium sulphate precipitations of a dilute sulphuric acid extract of the minced gland. Most of the work was carried out in a cold room until final crystallization, but as it continued over 5 or 6 days, proteolytic action could not be ruled out. An alternative method of preparation was therefore devised. The pancreas was taken immediately after slaughter and covered with solid carbon dioxide. The frozen glands were allowed to thaw sufficiently to be homogenized with 2 vol. 0·5M (8%, w/v) trichloroacetic acid in a Waring blender. This extraction was repeated and trichloroacetic acid was added to the combined filtrates to give a final concentration of 10% (w/v). The precipitate, which was readily soluble in water, contained most of the original ribonuclease activity. After dialysis against water in the cold it was freeze-dried. It seems unlikely that during this method of preparation any proteolysis could have occurred and from the activity:nitrogen ratio the material appeared to be about 30–50% pure. Fig. 4 shows the chromatogram obtained with this material and it can be seen that again the smaller component appears in the same position, though perhaps in a larger relative amount.

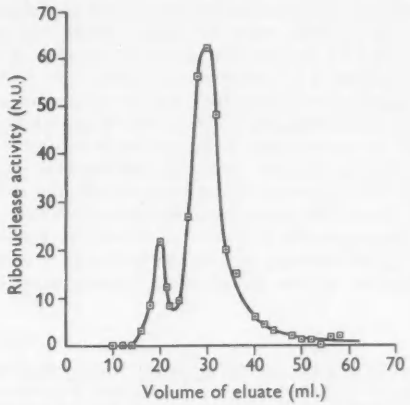

Fig. 4. Chromatogram of ribonuclease prepared by the trichloroacetic acid method. System: 20 g. $(NH_4)_2SO_4$, 20 g. cellosolve, 56 g. water; 6 g. silica column.

DISCUSSION

Comparison of the partition coefficient derived from the rate of movement of the bands (Martin & Synge, 1941) and that measured directly shows that the columns are not behaving simply as partition chromatograms. The bands move much more slowly than expected. (Expected $R = (a)$ 0·28, (b) 0·45. Found $R = (a)$ 0·08, (b) 0·30.) Since in the absence of the stationary phase the kieselguhr shows no adsorption, adsorption must be occurring in the interface between the two liquid phases. As might be expected, the discrepancy is greater, and hence adsorption also, with solvents of high salt content.

Perhaps related to this adsorption into the interface is the surprising efficiency of the separation, which is indeed about what would be expected with small molecules on the same columns, and this is in spite of the fact that the rate of flow of the liquid was certainly no slower than normal. If the bulk of the proteins were not in the interface, its low diffusion constant should cause low separation efficiency unless the flow rate was extremely small.

From the evidence presented it seems reasonable to conclude that ribonuclease exists in beef pancreas in two distinct forms; recrystallization failed to eliminate the smaller component, though if repeated often enough this might be possible. It has been shown previously that proteins with the same

specific biological activity when isolated from different species may differ chemically. The haemoglobins are an example of this (Porter & Sanger, 1948), and it is clear that some variation in structure may occur in such a series of compounds without the specific action being greatly modified. From solubility studies, Desreux & Herriott (1939) postulated the existence of at least two components in swine pepsin, both of which possess enzymic activity. It is probable that the duality of ox ribonuclease is a similar phenomenon. As a similar composition was found for a number of preparations made from small numbers of pancreases obtained over 12 months from different slaughter houses, it would seem that the two forms exist together rather than that individual animals have wholly one or the other type. The structural differences which alter the chromatographic behaviour have not yet been investigated and may well be subtle enough to escape detection by the technique at present available. From the aspect of protein structure these results would appear to give support to the idea of the existence of families of nearly identical proteins (cf. Synge, 1950).

SUMMARY

1. A partition chromatogram using the two liquid phases produced by mixtures of ammonium sulphate, ethylene glycol monoethyl ether ('cellosolve') and water has been devised with kieselguhr used as the inert support.

2. On such chromatograms crystalline ribonuclease gives without difficulty zones having convenient rates and symmetrical form. It was found to contain two enzymically active components in the approximate ratio 10:1.

3. Evidence is presented which suggests that both forms pre-exist in ox pancreas.

We wish to thank Mrs A. Dovey for her electrophoretic examination of ribonuclease.

REFERENCES

Desreux, V. & Herriott, R. M. (1939). *Nature, Lond.*, **144**, 287.
Herbert, D. & Pinsent, A. J. (1948). *Biochem. J.* **43**, 193.
Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
Kunitz, M. (1940). *J. gen. Physiol.* **24**, 15.
McDonald, M. R. (1948). *J. gen. Physiol.* **32**, 39.
Martin, A. J. P. (1949). Unpublished observations.
Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1558.
Porter, R. R. (1950). Unpublished observations.
Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.
Synge, R. L. M. (1950). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 191.

Inhibition of Alkaline Phosphatase by Oestradiol Phosphates

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In a preliminary report Aldman, Diczfalusy & Rosenberg (1948) have shown that kidney alkaline phosphatase is inhibited by some phosphoric acid esters of the oestrogenic steroids. In this paper more detailed data are presented as to the specificity, reversibility and mode of inhibition.

MATERIALS AND METHODS

Enzyme. The enzyme was prepared, as previously, from the kidneys of young rabbits, using with slight modifications the method described by van Thoai, Roche & Sartori (1944). In the course of repeated fractionations it was found that the enzymic activity of the crystalline material decreased simultaneously with increasing activity of the mother liquor, confirming thus the results of Abul-Fadl, King, Roche & van Thoai (1949), according to which the crystals consist of inorganic material with enzyme adsorbed on it. In some experiments electrophoretically purified enzyme has been used.

Substrates. The following substrates have been used: phenyl phosphate, β-glycerophosphate and glucose-6-phosphate (the last-named compound was kindly supplied by Dr O. Lindberg, Wenner-Gren Institute, Stockholm).

Analytical methods. Phenol was determined with Folin's reagent as described by Buch & Buch (1939), inorganic phosphate according to Lowry & Lopez (1946), and in some instances with a modified Martin & Doty (1949) method. The measurements were carried out in a Beckman spectrophotometer.

Conditions of enzyme experiments. Enzyme activity determinations were carried out within the pH range 7·0–11·0, using a 0·04 M-borate-carbonate-acetate buffer. Reaction volume, generally 2·5 or 5·0 ml.; incubation time, 10 or 16 min. at 37°. Enzyme concentration, 1·0 μg. of protein/ml., or less.

Inhibitors. The following substances were examined as to their inhibitory action: oestradiol 3-phosphate, oestradiol 17-phosphate, oestradiol 3:17-diphosphate and oestrone glucoside. Oestradiol 3-phosphate and oestradiol 17-phosphate were synthesized according to Miescher & Meystre (1946), oestradiol 3:17-diphosphate as described by Claesson, Högberg & Rosenberg (1949). Oestrone glucoside was synthesized by deacetylation of oestrone tetra-acetyl glucoside according to Kongsted (1939).

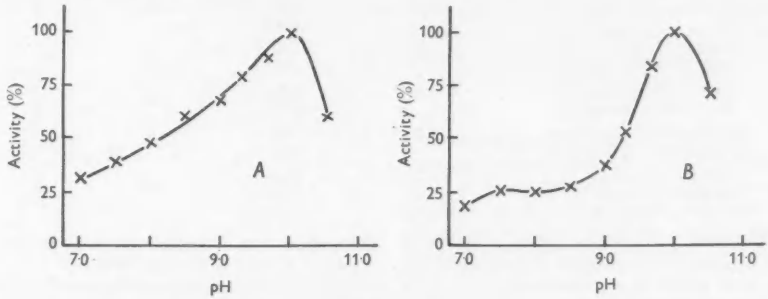

Fig. 1. Relationship between pH and phosphatase activity at different stages of purification. *A*, partially purified enzyme (identical with the initial product of van Thoai *et al.* 1944); *B*, highly purified enzyme (obtained after six reprecipitations by 50% (v/v) acetone). Activity is expressed as a percentage of the activity at the pH optima. Substrate, 0·005 M-phenyl phosphate. Acetate-carbonate-borate buffer, 0·04 M 16 min.; incubation at 37°.

RESULTS

The relationship between enzymic activity and pH is influenced by impurities as shown in Fig. 1, where the activity of a preparation at two different degrees of purity is plotted against pH. The activity is expressed as a percentage of the activity at the pH optima.

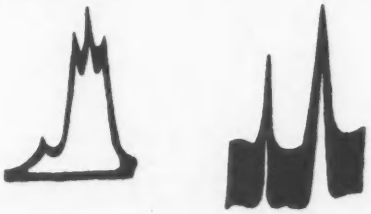

Fig. 2. Electrophoretic patterns of partially purified (*A*) and highly purified (*B*) kidney alkaline phosphatase. Acetate buffer, pH 5·9, ionic strength 0·1 at 0·5°. (*A*), 2·5 hr. electrolysis at 7·7 V./cm., 1·2% solution. (*B*), 3·5 hr. electrolysis at 7·9 V./cm., 0·5% solution.

It is of interest that preparation *B* has about 25% of the maximal activity even at pH 7·5. The less pronounced second peak at pH 7·5, shown in curve *B*, was invariably observed in experiments with highly purified preparations and phenyl phosphate as a substrate. The significance of this second peak is not clear at present. In electrophoretic studies, using acetate buffer at various pH values between 3·5 and 5·9 (ionic strength 0·1), it was revealed that the highly purified preparation showed the presence of only two components, one of which could be identified as the active principle. The partially purified preparation, examined at the same ionic strength and pH, contained at least four components (see Fig. 2).

From electrophoretic mobilities at different pH values the isoelectric point of kidney alkaline phosphatase was estimated to be approximately pH 4·8–4·9, as shown in Fig. 3.

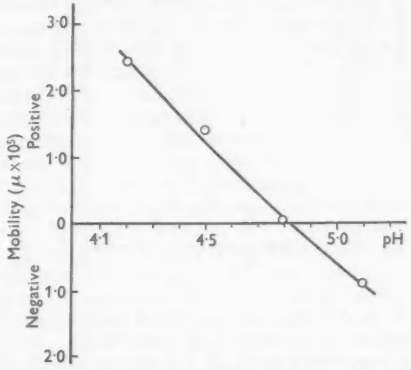

Fig. 3. Electrophoretic mobility of kidney alkaline phosphatase as a function of pH. Acetate buffer, ionic strength 0·1, temp. 0·5°. Highly purified enzyme, 0·35–0·5% solutions.

Inhibitors. The relative inhibitory powers of the various compounds at pH 9·3 are compared in Table 1. In the concentrations used monophosphates inhibit phosphatase only slightly. Among the different substances tested oestradiol diphos-

Table 1. *Inhibition of kidney alkaline phosphatase by some derivatives of the oestrogenic hormones*

(Acetate-carbonate-borate buffer, pH 9·3, 16 min. incubation at 37°. Substrate: 0·004 M-phenyl phosphate. Enzyme-protein concentration, 0·81 μg./ml.)

Inhibitor	Concn. (M)	Phosphatase activity (μg. phenol liberated) Individual exp.	Mean	Inhibition (%)
Nil	—	113	113	—
Nil		113		
Nil		114		
Oestradiol diphosphate	10^{-5}	70	70	38
Oestradiol diphosphate		69·5		
Oestradiol 3-phosphate	2×10^{-4}	107	107	5
Oestradiol 3-phosphate		108		
Oestradiol 17-phosphate	10^{-4}	92	91	19
Oestradiol 17-phosphate		90		
Oestrone glucoside	2×10^{-4}	114	115	0
Oestrone glucoside		117		

phate is the most powerful inhibitor. The same results are obtained in the presence of magnesium or manganese ions, which in a concentration of 10^{-3} M do not interfere with the inhibition by oestradiol diphosphate.

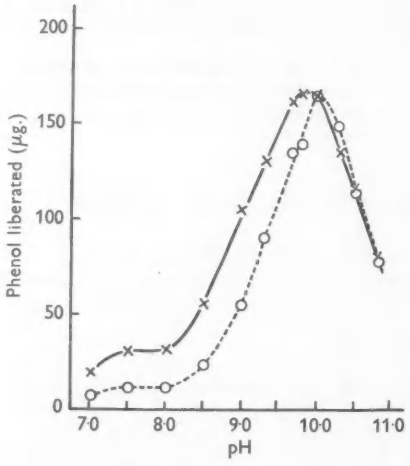

Fig. 4. Relationship between pH and inhibition of phosphatase by 10^{-5} M-oestradiol diphosphate. Acetate-carbonate-borate buffer, 37°, 16 min. incubation. Substrate, 0·004 M-phenyl phosphate. Enzyme protein concentration, 0·9 μg./ml. ×—×—×, without inhibitor; O---O, with 10^{-5} M-oestradiol diphosphate.

Type of inhibition. The inhibition of alkaline phosphatase by oestradiol diphosphate, using phenyl phosphate as a substrate, is presented in Fig. 4. The inhibition is limited to the acid side of the pH optimum, the greatest inhibition occurring at pH 7·5–9·0. An inhibition of kidney alkaline phosphatase of this type by various ions has been described by Aebi & Abelin (1948).

The inhibition is independent of the nature of the substrate used; with phenyl phosphate, β-glycerophosphate and glucose-6-phosphate, essentially the same results are obtained. In Fig. 5 the inhibition by oestradiol diphosphate of the enzymic hydrolysis of β-glycerophosphate by alkaline phosphatase is presented.

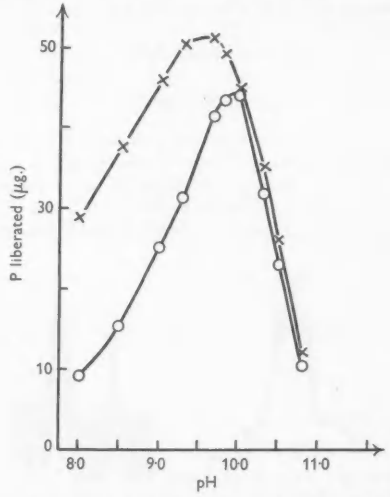

Fig. 5. Relationship between pH and inhibition of the enzymic hydrolysis of β-glycerophosphate by oestradiol diphosphate. Acetate-carbonate-borate buffer, 37°, 16 min. incubation. Substrate concentration, 0·005 M. Enzyme-protein concentration, 1·2 μg./ml. ×—×—×, without inhibitor; O—O—O, with 2×10^{-5} M-oestradiol diphosphate.

To test whether the inhibition was competitive or non-competitive, the concentrations of the substrate (phenyl phosphate) and inhibitor (oestradiol diphosphate) were varied at constant concentration of the enzyme. In Fig. 6 the reciprocal of the rate of

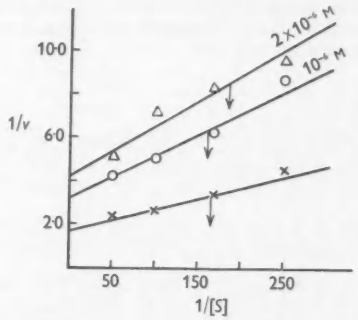

Fig. 6. Relation between enzyme activity and substrate concentration with different concentrations of oestradiol diphosphate. Activity is expressed as the reciprocal of the amount of P liberated in 10 min. at 37°. Enzyme-protein concentration: 0·81 μg./ml. Substrate: phenyl phosphate. Acetate-carbonate-borate buffer, pH 8·5. ×—×—×, without inhibitor; ○—○—○, 10^{-6} M-oestradiol diphosphate; △—△—△, 2×10^{-6} M-oestradiol diphosphate.

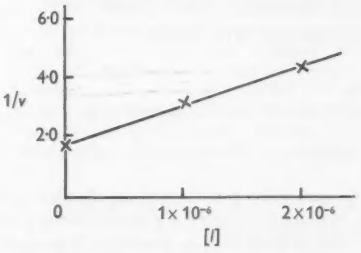

Fig. 7. Relation between enzyme activity and inhibitor concentration [I] at pH 8·5. Substrate: phenyl phosphate, acetate-carbonate-borate buffer, inhibitor: oestradiol diphosphate. 10 min. incubation at 37°. Enzyme activity expressed as the reciprocal of the rate of hydrolysis extrapolated for $[S] = \infty$. Enzyme-protein concentration: 0·81 μg./ml.

hydrolysis (v, expressed as μg. P liberated after 10 min. incubation at 37°) is plotted as ordinate against the reciprocal of the substrate concentration (S) following the procedure of Lineweaver & Burk (1934). It appears that the inhibition is not substrate-competitive, in contrast to what might be expected, since the inhibitor itself is a phosphoric acid ester. The dissociation constant, K_s, of the enzyme-substrate complex is 6×10^{-3}.

In Fig. 7 the reciprocal values of the rate of hydrolysis extrapolated for $[S] = \infty$ are plotted against the concentration of the inhibitor. It appears that the relationship is linear.

The curves in Figs. 6 and 7 are consistent with the equation

$$\frac{1}{v} = \frac{1}{v_{\infty(0)}}\left[1 + \frac{K_s}{[S]} + \frac{[I]}{K_I}\right],$$

where [I] is the concentration of the inhibitor, $v_{\infty(0)}$ is the rate of hydrolysis extrapolated for $[S] = \infty$ and $[I] = 0$, and K_I the dissociation constant of the complex of enzyme, substrate and inhibitor (ESI) according to the reaction $ESI \rightleftharpoons ES + I$.

$$K_I = 1{\cdot}4 \times 10^{-6}.$$

Reversibility of inhibition. The inhibition of the enzyme by oestradiol 3:17-diphosphate could be quantitatively reversed by small amounts of basic proteins, such as protamine sulphate (0·5 g./l.). These substances do not activate the alkaline phosphatase preparations used.

DISCUSSION

Since the inhibition of phosphatase by oestradiol diphosphate is non-competitive, it appears that the attachment between enzyme and inhibitor is not at the same place as the contact between enzyme and substrate. The reversibility, together with the non-competitive type of inhibition, may suggest a displacement of a possible coenzyme, suggested by others (Albers, Beyer, Bohnenkamp & Müller, 1938; Kutscher & Wüst, 1942; Abul-Fadl & King, 1949), though the evidence for the existence of such a coenzyme is doubtful (Schales & Mann, 1948). The most recent evidence of Kutscher & Sieg (1950) suggests that the prosthetic group of alkaline phosphatase is probably choline pyrophosphate. The complex of enzyme, substrate and inhibitor described in this paper may be analogous to the enzymically inactive complex of the enzyme with two molecules of substrate, described and discussed by others (Folley & Kay, 1935; Aebi, 1949), but the affinity of the enzyme-substrate complex for another substrate molecule is much less than the affinity of the complex for a molecule of the inhibitor (oestradiol diphosphate) as it appears from their respective dissociation constants. The corresponding standard free energies (37°) calculated from the dissociation constants are −430 cal. for the reaction $ES + S \rightarrow ES_2$, and −8400 cal. for the reaction $ES + I \rightarrow ESI$.

SUMMARY

1. Purified kidney alkaline phosphatase is partly inhibited by oestradiol 3:17-diphosphate in concentrations lower than 10^{-5} M. This inhibition is not

appreciably affected by the presence of magnesium or manganese ions.

2. The monophosphates of oestradiol are much weaker inhibitors than oestradiol 3:17-diphosphate, while oestrone glucoside does not exert any inhibitory effect in the concentrations investigated.

3. The inhibitory effect of oestradiol 3:17-diphosphate on alkaline phosphatases is greatest in the region of pH 7·5–9·3, and is not demonstrable on the alkaline side of the pH optimum.

4. The inhibition is independent of the nature of the substrate; using phenyl phosphate, β-glycerophosphate, or glucose-6-phosphate, essentially the same degree of inhibition is observed.

5. The inhibition of alkaline phosphatase by oestradiol 3:17-diphosphate is reversible and non-competitive.

REFERENCES

Abul-Fadl, M. A. M. & King, E. J. (1949). *Biochem. J.* **44**, 435.

Abul-Fadl, M. A. M., King, E. J., Roche, J. & Thoai, N.-van (1949). *Biochem. J.* **44**, 428.

Aebi, H. (1949). *Helv. chim. Acta*, **32**, 464.

Aebi, H. & Abelin, I. (1948). *Helv. chim. Acta*, **31**, 1943.

Albers, H., Beyer, E., Bohnenkamp, A. & Müller, G. (1938). *Ber. dtsch. chem. Ges.* **71**, 1913.

Aldman, B., Diczfalusy, E. & Rosenberg, T. (1948). *Acta chem. scand.* **2**, 529.

Buch, I. & Buch, H. (1939). *Acta med. scand.* **101**, 211.

Claesson, L., Högberg, B. & Rosenberg, T. (1949). Swedish Patent 126,564.*

Folley, S. I. & Kay, H. D. (1935). *Biochem. J.* **29**, 1837.

Kongsted, A. J. H. (1939). *Chem. Abstr.* **33**, 1449.

Kutscher, W. & Sieg, H. (1950). *Naturwissenschaften*, **37**, 451.

Kutscher, W. & Wüst, H. (1942). *Hoppe-Seyl. Z.* **273**, 235.

Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.

Lowry, O. H. & Lopez, J. A. (1946). *J. biol. Chem.* **162**, 421.

Martin, J. B. & Doty, D. M. (1949). *Anal. Chem.* **21**, 965.

Miescher, K. & Meystre, C. (1946). *Chem. Abstr.*, **40**, 179.

Schales, O. & Mann, G. E. (1948). *J. biol. Chem.* **175**, 487.

Thoai, N.-van, Roche, J. & Sartori, L. (1944). *C.R. Soc. Biol., Paris*, **138**, 47.

* No abstracts are yet available.

The Fate of Radioactive Potassium Chloride Administered Intraperitoneally or Intravenously to Rats

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(*Received* 1 *January* 1951)

It is known that when potassium salts are administered intravenously to animals the small increase found in the concentration of potassium in the serum can only be explained by assuming that the injected potassium is distributed in a volume of body water greater than that of the extracellular fluid. At least some of the cells of the body, therefore, are readily permeable to potassium. Hahn, Hevesy & Rebbe (1939), Joseph, Cohn & Greenberg (1939), Noonan, Fenn & Haege (1941), Fenn, Noonan, Mullins & Haege (1941) and Hevesy (1942) have studied the distribution of injected radioactive potassium salts. Noonan *et al.* (1941) administered radioactive potassium chloride intraperitoneally to rats and found that equilibrium between the extracellular and intracellular potassium of liver, heart, lung, kidney, gut and skeletal muscle was reached in 4–6 hr. The doses of potassium chloride varied from 6 to 59 mg. (0·3–4·1 m-equiv. potassium/kg.). Within 0·5–1 hr. after the injection, the liver, heart, lungs, kidneys and gut were so highly radioactive as to suggest that radioactive potassium had, at least, in part entered the cells as a result of a process other than exchange. They remarked that it was not possible, on the basis of their experiments, to separate the two processes.

In the experiments described below, the distribution of large doses of ^{42}KCl after a number of hours and of doses of 0·01 and 1 mg. of ^{42}KCl 5 min. after injection was studied.

METHODS

Female albino rats weighing between 192 and 230 g. were used.

The potassium salts used were irradiated KCl or K_2CO_3. The latter was neutralized to pH 8 by N-HCl and used in some experiments since the irradiation of KCl in the pile of the Atomic Energy Research Establishment, Harwell, yields ^{42}K and traces of ^{35}S and ^{32}P. We are grateful for information from Harwell that the activity due to NaCl present

as an impurity in the irradiated KCl accounted for about 0·1 % of the total radioactivity.

The decay curves of samples of ^{42}KCl followed the theoretical curve for pure ^{42}K during the 3 or 4 days following irradiation. This was the period during which the experiments were carried out, so that in calculating results, the half-life period of the radioactive material was taken to be 12·4 hr. The decay curve of $^{42}K_2CO_3$ followed the theoretical curve for 7–8 days, after which the two curves were different, an effect probably due to traces of rubidium present as an impurity in the K_2CO_3.

Four solutions of ^{42}KCl or neutralized $^{42}K_2CO_3$ in distilled water were prepared: *A*, 2 %; *B*, 0·2 %; *C*, 0·002 %; and *D*, 0·00002 %. *B*, *C* and *D* were obtained by diluting *A* with distilled water. *D* was used as a standard of ^{42}K activity and *A*, *B* and *C* were used for injections. The results to be described later in this paper were the same whichever solution was injected.

Two groups of experiments were performed. *Series* 1. Rats were taken from stock and injected intraperitoneally with radioactive material. After the appropriate time the rat was anaesthetized with sodium amytal (10 mg./100 g. rat, intraperitoneally), the carotid artery was dissected out and the animal bled for a period of about 1 min. into a centrifuge tube containing a trace of heparin. By this means about half the total amount of blood of the rat was usually obtained easily and without loss. The organs were quickly removed, weighed, and dissolved in hot concentrated HNO_3. Foaming was minimized by the use of an antifoam silicone grease smeared on the stirring rods. The digests were made up to a known volume using dilute HNO_3. *Series* 2. Rats were starved for 24 hr. but were allowed water. Each rat was then fed 2 ml. of milk by stomach tube and kept at 25°, and quiet. 1–2 hr. later, the animal was anaesthetized with sodium amytal and then injected either intraperitoneally or intravenously (the intravenous injection occupying 2–3 min.) with radioactive material. After 5 min. the animal was bled and the organs were analysed.

The radioactivity of the digests was determined by means of an M. 6 liquid counter (Veall, 1948). These and the counts obtained from a standard solution were corrected for decay since the time of injection. Usually, the counts on a sample were recorded to give a total of 4500 or more to reduce the error of counting. The specific gravity of the solution did not influence the rate of counting in the specific gravity range 1·00–1·30 met with in the solutions used.

Recovery experiments were carried out on a number of rats in both series. The carcases, after removal of a number of organs which were 'counted' separately, were dissolved in HNO_3. The fat which separated was extracted with ether, the extracts were washed with dil. HNO_3 and the combined aqueous phases were diluted to a known volume and then 'counted'.

Table 1 is a record of a typical experiment giving details of the experimental observations and of the method of calculation.

RESULTS

Series 1. Ten rats were given equiactive doses of 2 % potassium chloride in distilled water intraperitoneally. The amount of potassium chloride injected varied from 1 to 80 mg. (0·06–4·83 m-equiv. potassium/kg.). The animals were killed after 6, 24 and 48 hr. and the tissues analysed for radioactivity as described above.

Noonan *et al.* (1941) calculated, for each tissue, the ratio of ^{42}K/^{39}K in the tissue to that in the plasma, and called this the relative potassium activity (R.P.A.) of the tissue. Therefore, when the radioactive potassium in the extracellular fluid has reached equilibrium with the intracellular potassium, the R.P.A. is 1. The R.P.A. of the tissues of the rats in Series 1 were calculated, assuming values (obtained from other experiments) for the potassium content of the tissues studied. The findings of Noonan *et al.* (1941) were, in general, confirmed in that after 6–48 hr. the R.P.A. of the tissues examined was about 1 (Table 2), except that skeletal muscle had not quite reached equilibrium in 6 hr. The recovery of ^{42}K varied from 90 to 96 %.

Table 1. *Record of a typical experiment showing the radioactivities of various tissues after intravenous injection of* ^{42}KCl

(Rat no. 33 (body wt. 204 g.) starved for 24 hr. prior to being given 2 ml. of milk at 8.55 a.m. on 3 May 1950. At 10.45 a.m. 0·25 ml. of 10 % sodium amytal solution was injected intraperitoneally. At 11.00 a.m. 0·5 ml. radioactive KCl solution containing 1·0 mg. KCl was injected intravenously over 3 min. At 11.05 a.m. the rat was bled and the tissues removed, weighed, dissolved in HNO_3, and counted. Amount injected equivalent to 120,000 counts/min.; background count, 13/min. Volume counted, 8 ml.)

Organ	Wt. of organ (g.)	Vol. of diluted digest (ml.)	Counts/min. (uncorr.)	Counts/min. (corr.) to time of injection*	Counts/min. Total in organ (*a*)	Counts/min. *a* as percentage of injected counts	*a*/g. tissue as percentage of injected counts/min.
Liver	5·73	25	2,640	3,316	10,363	8·6	1·5
Kidney	0·78	25	1,850	2,271	7,096	5·9	7·6
Heart	0·69	25	860	1,038	3,243	2·7	3·9
Lung	1·08	25	644	781	2,440	2·0	1·9
Plasma	2·92	25	464	544	1,699	1·4	0·48
Residue	—	500	1,130	1,496	93,520	77·9	—
					Recovery	98·5 %	

* Allowance made for resolving time of counter, the background count, and radioactive decay.

Table 2. *Mean values of relative potassium activity* (R.P.A.) *6, 24 and 48 hr. after injection of radioactive potassium into rats*

(R.P.A. is the ratio of $^{42}K/^{39}K$ in the tissue to that in the plasma.)

Time (hr.)	Liver	Kidney	Heart	Muscle
6	0·85	0·96	1·04	0·63
	(1·46)	(1·23)	(1·33)	(1·02)
24	1·05	0·87	1·04	0·89
48	0·92	0·91	0·89	0·78

Figures in brackets are mean R.P.A. values for the 5 and 6·5 hr. experiments of Noonan *et al.* (1941).

The results, however, give no indication of the initial distribution of the potassium injected or of the mechanisms by which the final distribution is achieved. Noonan *et al.* (1941) determined the distribution of ^{42}K injected 30 min. previously, but they used quantities of potassium comparable with those normally present in all the extracellular fluid. Under these conditions transport of potassium ions into cells was probable. This was avoided in Series 2 in which very small quantities of potassium chloride, containing amounts of potassium approximately equal to 0·05 and 5% of the total extracellular potassium, were injected intraperitoneally or intravenously.

Series 2. The results reported in Table 3 were obtained from rats prepared as described earlier. This seemed to lessen the scatter of the results. The animals were anaesthetized with sodium amytal and then given 1·0 or 0·01 mg. of radioactive potassium chloride in 0·5 ml. of water intraperitoneally or intravenously into the jugular vein. After 5 min. the rats were bled and the organs removed and

Table 3. *Distribution of* ^{42}K *5 min. after intraperitoneal or intravenous injection of radioactive potassium into rats*

(Results are expressed as percentage (x) of total radioactivity. x is defined by the expression

$$x = \frac{\text{counts/min./g. tissue}}{\text{total injected counts/min.}} \times 100\ D,$$

where D is a correction factor allowing for decay. The irradiated material for Exps. 25–33 was K_2CO_3, neutralized as described above.)

Wt. of KCl injected in 0·5 ml. water (mg.)	Rat no.	Liver	Kidney	Heart	Lung	Plasma	Recovery (%)
				Intraperitoneal			
0·01	25	2·22	2·72	0·85	0·63	1·11	92·3
	26	2·45	2·00	1·14	0·52	1·27	92·0
	34	2·40	2·10	—	—	0·29	—
	35	1·22	1·40	—	—	0·25	—
1·0	27	2·32	3·58	1·74	0·82	0·35	98·4
	28	3·37	3·03	1·16	0·76	0·47	97·3
	29	3·46	3·55	1·84	1·08	0·52	102
	38	2·48	3·16	—	—	0·28	—
	39	2·92	2·35	—	—	0·24	—
	Means	**2·54**	**2·65**	**1·35**	**0·76**	**0·53**	—
				Intravenous			
0·01	30	1·53	7·65	4·79	2·57	0·29	99·6
	36	1·50	8·45	—	—	0·34	—
	37	1·72	7·91	—	—	0·36	—
	(23)†	1·41	7·33	4·63	2·11	0·35	93·6
1·0	31	1·66	8·16	5·53	2·22	0·58	103
	32	1·27	6·67	3·26	1·77	0·44	95·3
	33	1·51	7·62	3·89	1·89	0·49	98·4
	40	2·34	6·86	—	—	0·45	—
	41	1·44	7·54	—	—	0·42	—
	57	1·82	7·21	—	—	0·19	—
	58	1·79	10·95	—	—	0·32	—
	59	1·69	7·95	—	—	0·22	—
	60	2·25	9·28	—	—	0·27	—
	Means	**1·69**	**7·95**	**4·42**	**2·11**	**0·36**	—
	85*	1·63	7·95	—	—	0·52	—
	86*	2·53	10·10	—	—	0·65	—

* KCl solution injected made up in normal saline.

† Rat 23 had no preparation prior to injection.

analysed. When the injection of ^{42}KCl was given intraperitoneally, the abdominal organs were dipped in saline before analysis. Activities were calculated as percentage of injected radioactivity/g. of tissue, allowance having been made for radioactive decay.

Observations common to experiments in which intraperitoneal or intravenous injections were administered (Table 3)

Although the doses given were 0·01 and 1·0 mg. KCl, for a given route of injection, the uptake of ^{42}K in 5 min. by the tissues was not widely different.

The activities of the tissues were high 5 min. after injection, far higher than can be accounted for by the extracellular fluid present.

The radioactivity of the plasma showed wide variations. The experiments which have been bracketed were carried out on the same day. It will be observed that the plasma activities of these pairs of experiments agree closely but we are unable to account for the fact that the plasma activities varied widely on different days.

Recovery of injected ^{42}K ranged from 92 to 103 %.

Results of intraperitoneal injections (Table 3)

The activities per g. of liver and kidney were high and of the same order.

The plasma activities varied from 0·24 to 1·27 %.

Results of intravenous injections (Table 3)

The lung, heart and kidney showed very high uptakes of radioactive potassium in comparison with the corresponding uptakes observed after intraperitoneal injection.

The activity of the plasma varied from 0·19 to 0·58 %, but did not show as large a variation as in the corresponding series in which the dose was given intraperitoneally, although the orders of activity were similar.

The activity of the liver was about half, and that of lung, heart and kidney about three times that observed after intraperitoneal injection.

The very high activity of the lungs, heart and kidneys was not due to the hypotonicity of the injected fluid. The results obtained from rats 85 and 86 in which the injected fluid was ^{42}KCl in 0·9 % sodium chloride were identical with those obtained when aqueous solutions of ^{42}KCl were injected.

DISCUSSION

Five minutes after intraperitoneal injection of ^{42}KCl the tissues of the intestine contained 4–5 % of the injected ^{42}K/g. After intravenous injection, this figure was 2·5–3 %, a change comparable with that which occurred in liver. In similar experiments, the gastrocnemius muscle contained less than 0·3 % of the injected ^{42}K/g. whether the injection was given intraperitoneally or intravenously.

Previous workers have established the general distribution of injected potassium in rats after periods varying up to 22 hr., and reached the conclusion that the element was then distributed according to the distribution of body potassium. In our experiments, the doses of potassium salts varied more widely than in the experiments of Noonan *et al.* (1941), but the final distribution of the injected material appeared to be the same; that is, according to the distribution of body potassium.

The phrase 'transport of potassium' is used in this paper to mean the entry of potassium ions with anions into the cells because of osmotic requirements and as a mechanism for the removal of an excess of potassium ions from the extracellular fluid. Exchange operates as a result of a dynamic equilibrium between intra- and extra-cellular potassium ions. Hence, if a quantity of radioactive potassium, small in amount (say, less than 5 %), compared with the total potassium in the extracellular fluid were injected, any entry of ^{42}K into the tissues should be the result of ionic exchange, and not of transport.

On the assumption that the total extracellular fluid in a rat of 200 g. is 35–40 ml., the extracellular potassium would amount to about 7–8 mg. Thus, the amount of ^{42}K injected in the experiments referred to in Table 3 could not increase the total extracellular potassium by more than 0·06 and 6 %. Neither increase is likely to lead to transport of potassium into the cells. In spite of this, liver, heart, lung and kidney were highly radioactive 5 min. after the injection. This must be ascribed to a process of rapid exchange between intra- and extra-cellular potassium ions and indicates a considerable degree of permeability of these cells to potassium. Within 5 min. of injection the gastrocnemius muscle, on the other hand, showed scarcely any activity, so that the surmise of Noonan *et al.* (1941, p. 487) 'that it is possible that exchange is no more rapid in liver than in muscle' is not supported.

The rate at which the injected solution leaves the peritoneal cavity is proportional to the hypotonicity of the solution (Cunningham, 1926; Orlow, 1895). The solutions of 0·2 and 0·002 % potassium chloride used in our experiments were very rapidly absorbed, about 50 % of the active material leaving the peritoneal cavity in the first minute or so. In 5 min. 90 % was absorbed. The absorption of active potassium from the somewhat hypertonic solutions used by Noonan *et al.* (1941) would depend partly upon exchange and partly upon the rate at which the fluid injected could be converted into a plasma ultrafiltrate. This would involve transport of potassium into the portal blood. That these pro-

cesses of transport and of exchange are fairly rapid is shown by the statement by these workers that only about 15% of active material remained in the peritoneal cavity after 10 min.

Since the order of radioactivity of the plasma following intravenous and intraperitoneal injection is practically the same, the two sets of experiments are comparable. The notable differences concern the activities of the liver, kidney, heart and lung. The increase in activity of the kidney, heart and lung when the solution was injected intravenously over that found after intraperitoneal injection was roughly in the same proportion for each organ. It can be calculated that because of their favourable anatomical positions when the intraperitoneal route is used for injection the liver and gut remove, by exchange, some 40–50% of the active potassium injected during the passage of the highly radioactive blood through these tissues for the first time. This must indicate therefore a very high exchange rate.

After intraperitoneal injection it is probable that absorption of potassium occurs mainly into the portal blood, so that the liver cells are perfused with blood rich in ^{42}K and, being readily permeable to potassium ions, a large proportion of ^{42}K from the perfusing blood exchanges with potassium of the liver cells. The blood in the hepatic vein would have, therefore, a lower radioactivity than the portal blood. The cells of the lungs, heart and kidney, though readily permeable to potassium ions, are perfused with blood of lower activity than the liver and so their opportunity for acquiring ^{42}K by exchange is less. The liver is therefore anatomically the most favourably situated of the organs mentioned and this would account for its high radioactivity. When the injection is given intravenously, however, the liver is least favourably situated, since only a part of its blood supply is derived from arterial blood rich in ^{42}K. The portal venous blood has already traversed the gut, the cells of which are readily permeable to potassium ions. This interpretation accords with the differences shown in Table 3. No attempt has been made to calculate the extent of the exchange which has occurred in the experiments lasting 5 min., because the activity of the plasma leaving the different organs was not known. The values of plasma activity given in Table 2 cannot be assumed to represent the activity of the venous outflow from any particular organ. In experiments of short duration, therefore, R.P.A. values are misleading.

SUMMARY

1. The distribution of 1–80 mg. of potassium chloride injected intraperitoneally after 6, 24 and 48 hr. has been studied using radioactive potassium (^{42}K) as a tracer. The distribution at equilibrium is according to the location of the body potassium for the tissues analysed.

2. The distributions of 1·0 and 0·01 mg. of radioactive potassium chloride administered intraperitoneally or intravenously 5 min. previously are given and the differences discussed.

3. Evidence is presented that there is a rapid exchange between the extra- and intra-cellular potassium of the liver, gut, kidney, heart and lung, but it is slow for the gastrocnemius muscle.

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REFERENCES

Cunningham, R. S. (1926). *Physiol. Rev.* **6**, 242.
Fenn, W. O., Noonan, T. R., Mullins, L. J. & Haege, L. (1941). *Amer. J. Physiol.* **135**, 149.
Hahn, L. A., Hevesy, G. & Rebbe, O. H. (1939). *Biochem. J.* **33**, 1549.
Hevesy, G. (1942). *Acta physiol. scand.* **3**, 123.
Joseph, M., Cohn, W. E., Greenberg, D. M. (1939). *J. biol. Chem.* **128**, 673.
Noonan, T. R., Fenn, W. O. & Haege, L. (1941). *Amer. J. Physiol.* **132**, 612.
Orlow, W. N. (1895). *Pflüg. Arch. ges. Physiol.* **59**, 170.
Veall, N. (1948). *Brit. J. Radiol.* **21**, 347.

The Influence of Pyridoxine on Fat Metabolism in the Rat

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The role of pyridoxine in fat metabolism has been the subject of a number of investigations. McHenry & Gavin (1938) reported that supplementation of a synthetic diet with a concentrate of rice polishings together with vitamin B_1, riboflavin and choline resulted in a greater deposition of body fat than occurred with vitamin B_1 and riboflavin alone. The inclusion of rice concentrate of rice polishings provided a good supply of pyridoxine. Each of these factors appeared to contribute to the increase of body fat, the effect being, at least in part, independent of appetite stimulation. In later work these authors (McHenry & Gavin, 1941) observed that rats on a basal diet containing 96% caseinogen developed more body fat when receiving supplements of vitamin B_1, riboflavin, pantothenic acid, choline, nicotinamide and pyridoxine than when pyridoxine was omitted. They interpreted their results on the hypothesis that pyridoxine is required for the synthesis of fat from protein, and suggested that it is necessary for such stages of protein metabolism as may be essential for the conversion of protein into carbohydrate. Sure & Easterling (1949) compared the body fat of groups of rats maintained for 26–63 days on diets with and without pyridoxine with that of weanling animals fed for 1 week on a stock diet. A paired-feeding technique was employed in these experiments. During the experimental period the pyridoxine-deficient animals suffered a mean loss of 0·24 g. fat, whereas those receiving pyridoxine showed a mean gain of 8·12 g. fat.

Many factors are known to influence the level of fat in the liver. Halliday (1938) claimed that there was an accumulation of fat in the livers of rats rendered deficient in pyridoxine. The vitamin supplements used in these experiments were, however, incomplete, and the food intakes of the normal and deficient animals were not controlled. Engel (1942) found that, with rats subsisting on an unrestricted diet, deprivation of pyridoxine for a period of 6 weeks was without effect on the liver fat. If, however, the deficiency were prolonged up to 8–24 weeks, the deficient animals had a liver-fat content approximately twice that of the controls. The use of dry ethyl ether in these experiments for extraction of the fat is open to objection and may have been responsible for unduly low values. Thus, Erlandsen (1907) pointed out that ether is an inefficient solvent for tissue lipids unless the tissue had undergone preliminary treatment with ethanol. Engel's findings are further complicated by a coincident deficiency in the diets used of inositol and biotin, both of which are known to influence the level of liver fat. On the other hand, Gavin & McHenry (1940) have concluded that pyridoxine has no supplementary action with choline in preventing the deposition of fat in the liver which occurs when vitamin B_1 is administered. The importance of controlling the food intake so as to eliminate the effects of variations of appetite was emphasized by Quackenbush, Steenbock & Platz (1942). These workers observed that, in acrodynic rats deficient in pyridoxine and pantothenic acid, the addition of rice-bran concentrate supplying both these factors induced a marked increase of liver and body fat if the basal diet was supplied *ad lib.* If, however, the body weights of the control animals were maintained constant by restriction of food intake, no increase of fat occurred as a result of supplementation with rice-bran.

In the present experiments the neutral fat, phospholipin and total cholesterol content of the liver, and the total body lipids have been determined in rats subjected to uncomplicated deficiency of pyridoxine. In addition, observations are reported on the oxidation of fatty acids by liver tissue, and on the faecal excretion of fat. The food intake of control animals has been equated to that of the deficient animals by a paired-feeding technique. The food consumption of each deficient animal was determined daily. This amount of food was fed to the corresponding control receiving pyridoxine in the succeeding 24 hr. In one series, animals fed by this technique were compared with normal and deficient animals which were fed *ad lib.*

METHODS

Diet. Two types of basal diet, of low and high fat content respectively, have been employed. These diets had the following composition:

	Low-fat diet (g.)	High-fat diet (g.)
Casein	18	20
Sucrose	63·5	50
Salt mixture	4·5	5
Cod liver oil	4	5
Water	10	—
Margarine	—	20

Casein used in these diets was twice extracted with 50% ethanol, followed by two extractions with 96% ethanol, over a period of a week with frequent stirring. The salt mixture had the following composition: NaCl, 4·69 g.; $MgSO_4.7H_2O$, 7·22 g.; Na_2HPO_4, 9·21 g.; KH_2PO_4, 25·82 g.; $Ca_3(PO_4)_2$, 14·62 g.; calcium lactate, 35·22 g.; ferric ammonium citrate, 3·15 g.; $CuSO_4.5H_2O$, 0·05 g.; KI, 0·01 g. Food consumption of the deficient animals was determined daily and the controls were pair-fed. All rats received daily supplements of vitamin B_1, 20 μg.; riboflavin, 40 μg.; nicotinamide, 100 μg.; calcium D-pantothenate, 240 μg.; *p*-aminobenzoic acid, 250 μg.; biotin, 2·5 μg.; inositol, 500 μg.; choline chloride, 1 mg. and arachis oil, 3 drops. Riboflavin, calcium D-pantothenate, inositol, *p*-aminobenzoic acid, nicotinamide and biotin from Roche Products Ltd. The controls received in addition pyridoxine hydrochloride 40 μg. daily. The animals were killed by stunning and decapitation when typical signs of dermatitis were manifest.

Fat estimations. Total body lipids were estimated by refluxing the whole carcass with 30% (w/v) ethanolic KOH for 6 hr. After cooling, the solution was transferred to a 500 ml. flask and made up to volume with 96% (v/v) ethanol. Duplicate 50 ml. portions were pipetted into evaporating dishes, acidified with conc. HCl and evaporated to dryness on a water bath. The residues were transferred to Soxhlet thimbles and extracted twice with ethyl ether for 72 hr. The second extraction yielded negligible amounts of fat. The ethereal extracts were evaporated to dryness, shaken with $CHCl_3$ and filtered into weighed glass dishes. After evaporation to dryness in air, the $CHCl_3$-soluble material was dried in a vacuum desiccator to constant weight.

The following procedure was adopted for the determination of the liver lipids and cholesterol. The liver was weighed and a portion reserved for determination of dry weight. The remainder was transferred to a mortar containing about 5 vol. 97% (v/v) ethanol, minced with scissors and ground with sand. The supernatant was separated by centrifuging, and the residue re-extracted with about 50 vol. of a mixture at boiling temperature containing 97% (v/v) ethanol 2 parts and ethyl ether 1 part, used in eight portions. The final residue was continuously extracted in a Soxhlet apparatus for 6–8 hr. with ethyl ether. The combined extracts were evaporated nearly to dryness under reduced pressure below 50°. The residue was taken up in 40 ml. boiling light petroleum (A.R., b.p. 40–60°) in four or five portions and centrifuged at 4000 rev./min. The supernatant was decanted into a standard flask and made up to volume. The completeness of the extraction may be assessed from the fact that further extraction of the residue for 6 hr. with the ethanol-ether mixture yielded an additional 1–2% of light petroleum-soluble material.

The phosphorus content of the light petroleum extract was estimated by the method of Berenblum & Chain (1938). In calculating the phospholipin content, it was assumed that the phospholipins of rat liver contain 4% P.

Neutral fat was estimated by the dichromate oxidation method of Bloor (1928, 1929). Phospholipin was precipitated from a portion of the light petroleum extract with acetone and $MgCl_2$. The completeness of the precipitation was found to vary between 88 and 100% with a mean value of 96%. Thus, the acetone-soluble fraction contains, in addition to neutral fat and cholesterol, a small variable proportion of phospholipin. In order to determine the oxidation equivalent of neutral fat in terms of standard dichromate solution it is therefore necessary to make a correction for the oxidation equivalents of cholesterol and acetone-soluble phospholipin present in the acetone-soluble extracts.

The cholesterol content of the acetone-soluble fraction was estimated as described below, and Bloor's conversion coefficient (3·92) used in calculating its oxidation equivalent.

The acetone-soluble phospholipin was determined by estimation of phospholipin P as described above. In agreement with the results of Gortner (1945), the ratio phospholipin P/oxidation equivalent was found to vary in different experiments. In consequence, this ratio was determined in each experiment. The acetone-insoluble fraction was used for this purpose, and the assumption has been made that the acetone-soluble phospholipin is a representative sample of the phospholipin present in the original light petroleum extract.

In calculating the neutral fat, Bloor's conversion coefficient (3·60) was used. It was shown that, after addition of neutral fat to the light petroleum extract, a 97% recovery was obtained in the acetone-soluble fraction.

Cholesterol was estimated by the Liebermann-Burchard reaction, using the method of Sackett (1925), except that the reaction mixture was incubated in the dark for exactly 60 min. Standard solutions of cholesterol were estimated simultaneously, using a Spekker absorptiometer. The acetone-soluble fraction was used in these estimations. This procedure has two advantages. First, the cholesterol content of this fraction is required in calculating the neutral fat value. Secondly, it was found that the brown colour which occasionally interferes with the estimation of cholesterol by the Liebermann-Burchard method (Sperry & Brand, 1943) does not develop if the acetone-soluble fraction is used instead of the original light petroleum extract containing the bulk of the phospholipins. The recovery of added cholesterol averaged 99% when estimated colorimetrically, and 95% by the oxidation method. Prior hydrolysis of cholesteryl esters, as recommended by Sperry & Brand (1943), was found not to influence the results and was therefore omitted. This omission is not necessarily justified under experimental and dietary conditions differing from those adopted in the present experiments.

For the estimation of faecal fat, the faeces were collected over an 8-day period and allowed to dry in air at room temperature. The dried material was then ground to a fine powder and continuously extracted with ethyl ether in a Soxhlet apparatus for 10 hr. The ethereal extract was evaporated to dryness and the residue taken up in $CHCl_3$. The $CHCl_3$ extract was filtered, evaporated to dryness and dried in a desiccator to constant weight.

Oxidation of fatty acids by liver. The oxidation of sodium butyrate and sodium octanoate was studied by the Warburg technique, using liver slices from animals fasted overnight. The slices, having a thickness of 0·2–0·4 mm., were cut with a razor blade and triplicate samples transferred to Warburg bottles containing sodium β-glycerophosphate buffer (Jowett & Quastel, 1935) at pH 7·4 in the main chamber. The substrate, dissolved in buffer to give a final concentration of 0·02-M after tipping, was placed in the side arm. O_2 consumption was measured in an atmosphere of O_2 over a period of 90 min.

RESULTS

Effect of deprivation of pyridoxine on total body lipids

Table 1 records the values for total body lipids of normal and pyridoxine-deficient rats fed on basal diets having a low and high fat content respectively. The data are calculated on the basis of the lipid content/100 g. body weight. The deficient animals were deprived of pyridoxine for periods varying from 46 to 183 days in individual cases, and at the time when they were killed well marked signs of dermatitis were manifest. The data in Table 1 indicate that, in animals maintained on a diet low in fat, deficiency of pyridoxine results in a marked reduction of body fat as compared with the positive controls. This is the case whether the results are calculated on a percentage basis or whether the absolute levels of body fat are compared. This observation is in contrast to the results obtained with animals whose diet contained 20% fat (groups *C* and *D*). In this case the concentration of total body lipids of the deficient animals was not significantly less than that of animals receiving pyridoxine. When comparison is made between groups *A* and *C* it is evident that both the absolute amount and the concentration of body fat of deficient animals on the high-fat diet is significantly greater than that of deficient animals on the low-fat diet. On the other hand, the control groups in both series which received pyridoxine have a concentration of body fat which is independent of the level of preformed fat in the diet.

Effect of deprivation of pyridoxine on liver lipids

Table 2 summarizes the results of estimations of neutral fat, phospholipins and total sterols of pyridoxine-deficient rats and controls at low and high

Table 1. *Total body lipid content of rats deficient in pyridoxine*

Groups	Diet	No. of animals	Mean final body wt. (g.±S.E.)	Mean total body lipids (g./100 g. body wt.±S.E.)
A	Low-fat	5	75± 5	2·32±0·55
B	Low-fat + pyridoxine 40 μg.	5	114±13	8·55±1·20
C	High-fat	7	80± 7	5·17±1·02
D	High-fat + pyridoxine 40 μg.	7	122± 9	7·95±1·18

Statistical analysis of results

	AB	*AC*	*BD*	*CD*
t	4·70*	2·40*	0·35	1·78
P	<0·01	<0·05	0·8	0·1

* Denotes a significant difference.

Table 2. *Neutral fat, phospholipin and total sterol content of livers of pyridoxine-deficient and control rats*

Group	Diet	No. of animals	Final body wt. (g.)	Liver wet wt. (g.)	Liver water (g./100 g. liver)	(g./100 g. liver wet wt.±S.E.) Neutral fat	Phospholipins	Total sterols
E	Low-fat; pair-fed	12	88	3·97	71·2	0·63±0·11	3·66±0·19	0·28±0·03
F	Low-fat; pair-fed + pyridoxine 40 μg.	12	142	5·92	68·8	1·27±0·15	3·73±0·21	0·31±0·03
G	Low-fat; pair-fed	6	92	4·08	71·1	0·64±0·10	3·64±0·20	0·31±0·04
H	Low-fat; pair-fed + pyridoxine 40 μg.	6	141	5·55	69·6	1·31±0·18	4·00±0·15	0·31±0·04
I	Low-fat; fed *ad lib.* + pyridoxine 40 μg.	6	175	7·94	70·2	1·46±0·30	3·67±0·19	0·28±0·03
J	High-fat; pair-fed	3	107	4·53	68·9	0·93±0·14	3·87±0·08	0·31±0·03
K	High-fat; pair-fed + pyridoxine 40 μg.	3	151	4·93	68·7	1·99±0·20	3·33±0·26	0·31±0·02

Statistical analysis of results

	EF		*GH*		*GI*		*HI*		*JK*	
	t	*P*	*t*	*P*	*t*	*P*	*t*	*P*	*t*	*P*
Neutral	3·44*	<0·01	3·22*	<0·05	2·58*	>0·01	0·43	0·7	4·35*	<0·05
Phospholipin	0·25	>0·05	1·44	>0·05	0·11	>0·05	1·37	>0·05	1·98	>0·05
Sterol	0·71	>0·05	0·00	>0·05	0·60	>0·05	0·60	>0·05	0·00	>0·05

* Denotes a significant difference.

Table 3. *Faecal excretion of nitrogen in pyridoxine deficiency*

No. of observations	Mean nitrogen intake (mg./day)	Mean faecal nitrogen excretion (mg./day ± S.E.)		t	P
		Pyridoxine-deficient	Control		
27	148·6	9·3 ± 0·43	8·7 ± 0·54	0·87	<0·01

levels of dietary fat. In groups *G*, *H* and *I*, comparison is made between the liver lipids of deficient animals, pair-fed controls and controls fed *ad lib.*

The data for groups *E* and *F* and for groups *G* and *H* indicate that the percentage of neutral fat present in the livers of deficient animals on the low-fat diet is only about half that in the livers of the pair-fed controls. In the case of groups *J* and *K* relating to animals fed on the high-fat diet a similar difference in neutral fat is found for the livers of deficient and control animals. This result may be contrasted with the influence of preformed dietary fat on the total body lipid content of deficient animals already referred to. The percentages of neutral fat in the livers of animals fed on the high-fat diet are somewhat higher than those of the corresponding groups on the low-fat diet, but the differences are not significant. When comparison is made between pair-fed animals and controls fed *ad lib.* (groups *G*, *H* and *I*) it will be seen that the percentage of neutral fat in the livers of the latter is not significantly different from that of the pair-fed control, though in both cases the values are higher than that for the deficient group *G*.

Consideration of the values obtained for phospholipins and total sterols leads to the conclusion that the concentration of these constituents in the liver is not influenced by the absence of pyridoxine in the diet.

Effect of deprivation of pyridoxine on the absorptive function of the intestine

In the course of a study of the nitrogen balance in pyridoxine deficiency carried out for another purpose, estimations of faecal fat and faecal nitrogen were performed. The results, in so far as they relate to the problem of fat absorption in this condition, are as follows. In seven observations on animals on the high-fat diet, the mean total lipid content of the faeces in pyridoxine deficiency was 0·019 g./day compared with 0·017 g./day for control animals, the mean dietary intake of lipid being 0·89 g./day. The results indicate that there is no impairment of fat absorption as a result of deficiency of pyridoxine. Table 3 records data on the excretion of faecal nitrogen in normal and deficient animals receiving controlled intakes of protein. The balances were carried out over 8-day periods on animals at varying stages of depletion. The methods employed in these experiments will be described more fully in a later paper. Table 3 indicates that the difference in the mean values for faecal nitrogen between the deficient animals and controls was less than 1% of the nitrogen intake and was not significant. We are

Table 4. *Absorption of glucose by loops of jejunum and ileum in pyridoxine deficiency*

Exp. no.		Absorption of glucose (mg./cm. intestine/hr.)	
		Pyridoxine-deficient	Control
1	Jejunum	3·3	2·3
	Ileum	2·7	1·3
2	Jejunum	2·3	2·0
	Ileum	1·5	1·0

indebted to Dr R. B. Fisher and Dr D. S. Parsons for permission to include in Table 4 the results of experiments on the absorption of glucose by loops of small intestine from two pairs of rats by means of their *in vitro* perfusion technique (Fisher & Parsons, 1949). After making allowance for differences in length of the small intestine, the total absorptions of glucose per hr. by the deficient animals were 205 and 184 mg., whereas the corresponding values for

Table 5. *Oxidation of sodium butyrate and sodium octanoate by liver slices from normal and pyridoxine-deficient rats*

(Warburg bottles contained 2·5 ml. sodium β-glycerophosphate buffer pH 7·4 with 40–50 mg. liver slices in the main chamber and 0·5 ml. 0·12 M-substrate in the side arm. In measuring the residual respiration the substrate was replaced by 0·5 ml. buffer. The bottles were filled with O_2 and shaken at 38° for 90 min. CO_2 was absorbed by filter paper moistened with 30% (w/v) KOH in the centre well.)

No. of observations		Substrate	Final body wt. (g. ± S.E.)	Liver wet wt. (g. ± S.E.)	O_2 uptake (μl./hr./g. liver wet wt. ± S.E.)	t	P
5	Pyridoxine-deficient	Sodium butyrate	84 ± 15	3·48 ± 0·2	64 ± 22·3	6·0	<0·01
	Control	Sodium butyrate	110 ± 17	4·87 ± 1·6	309 ± 34·3	—	—
5	Pyridoxine-deficient	Sodium octanoate	59 ± 5	2·23 ± 0·03	207 ± 128	2·3	0·05
	Control	Sodium octanoate	104 ± 12	4·23 ± 0·39	696 ± 167	—	—

the controls were 209 and 206 mg. respectively. The results indicate that there is no overall impairment in the absorption of glucose by the pyridoxine-deficient rat.

Effect of deprivation of pyridoxine on the oxidation of sodium butyrate and sodium octanoate by liver slices

Table 5 summarizes data relating to the respiration of liver slices from pyridoxine-deficient and control animals fed on the low-fat diet with sodium butyrate and sodium octanoate as substrates. With both substrates, after correcting for the residual respiration, there is a significant reduction in oxygen uptake with liver slices from the pyridoxine-deficient group.

DISCUSSION

The failure of rats fed on a low-fat diet deficient in pyridoxine to deposit fat in the body, which is indicated in the present work, confirms the observation of Sure & Easterling (1949) who also adopted a paired-feeding technique in their experiments. The reduction of total body fat is associated with a lower value of the neutral fat content of the liver, though without change in the phospholipin and sterol fractions. Pyridoxine cannot, therefore, be regarded as a lipotropic agent. The absence of fatty livers cannot be due to lack of available fat to infiltrate the organ, since this condition was not observed even when the diet contained 20 % margarine. The reason for the fatty livers reported by Halliday (1938) and by Engel (1942) is uncertain; it may be noted that the degree of infiltration observed by these authors was comparatively slight, and that the diets employed were lacking in certain factors which are now believed to influence the content of fat in the liver. Our observations are in accord with those of Deane & Shaw (1947) who found that the livers of rats depleted of pyridoxine 'were uniformly free of histologically demonstrable lipid'.

The explanation for the low values of body fat in pyridoxine deficiency has not been hitherto examined. We have considered that there may be some failure in the absorptive function of the small intestine. Our evidence does not support this possibility and there seems, therefore, no reason to believe that disturbance in digestion or absorption of foodstuffs can account for the relative absence of body fat in this condition.

The reduction in the fatty acid oxidase activity of liver slices from pyridoxine-deficient rats, taken in conjunction with the reduction in substrate concentration revealed by the analyses of liver lipids, disposes of the possibility that a low value of body fat is a consequence of accelerated oxidation of fat.

There remains the hypothesis that in pyridoxine deficiency there is a failure of fat synthesis. Stetten & Boxer (1944) have demonstrated that in the well nourished rat 30 % of the ingested carbohydrate is stored as fat and only about 3 % as glycogen. In this connexion the determinations of body fat in animals fed on the high-fat diet are of interest. In the case of animals receiving pyridoxine the level of body fat is unaffected by the partial replacement of carbohydrate by fat in the diet. The fat present in the depots of such animals, and representing a mixture of preformed dietary fat and fat newly synthesized from carbohydrate, is fairly constant in relation to body weight. On the other hand, if pyridoxine deficiency involves an impairment in the synthesis of fat from carbohydrate, the provision of preformed fat in the diet might be expected to raise the level of body fat to that found in the animals receiving pyridoxine. The data recorded in Table 1 indicate that this is the case. It may be noted, however, that the level of neutral fat in the liver is not restored; this finding might be anticipated in the light of the observation of Frazer (1943) that dietary glyceride is transported to the depots rather than to the liver. The synthesis of fat in the animal body may, however, depend on the availability of other nutritional factors in addition to pyridoxine. Thus, Quackenbush & Steenbock (1942) observed that the depletion of liver and body lipids which occurred on a diet deficient both in pyridoxine and pantothenic acid was not restored on addition of pyridoxine alone to the diet. With both supplements, on the other hand, there was a threefold increase in body lipid and a marked rise in body weight. While the data presented in this paper are consistent with the hypothesis of a failure in fat synthesis as a result of pyridoxine deficiency, there is at present no evidence to elucidate the relevant enzyme systems which are affected.

SUMMARY

1. Deficiency of pyridoxine in the rat involves a lowering in the total lipid content of the body and a diminution in the neutral fat content of the liver. There is no change in the phospholipin or sterol fractions of the liver lipids in this condition. Inclusion of 20 % fat in the diet restores the total body lipid content to the level of that found in animals receiving an adequate intake of pyridoxine.

2. A decreased rate of oxidation of sodium butyrate and sodium octanoate by liver slices from pyridoxine-deficient rats is reported.

3. There is no impairment in the absorption of carbohydrate, fat or the products of protein digestion in pyridoxine deficiency

4. The observations reported are consistent with the view that deficiency of pyridoxine involves a failure in the ability of the rat to synthesize fat from carbohydrate.

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REFERENCES

Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Bloor, W. R. (1928). *J. biol. Chem.* **77**, 53.
Bloor, W. R. (1929). *J. biol. Chem.* **82**, 273.
Deane, H. W. & Shaw, J. H. (1947). *J. Nutrit.* **34**, 1.
Engel, R. W. (1942). *J. Nutrit.* **24**, 175.
Erlandsen, A. (1907). *Hoppe-Seyl. Z.* **51**, 71.
Fisher, R. B. & Parsons, D. S. (1949). *J. Physiol.* **110**, 281.
Frazer, A. C. (1943). *J. Physiol.* **102**, 306.
Gavin, G. & McHenry, E. W. (1940). *J. biol. Chem.* **132**, 41.
Gortner, W. A. (1945). *J. biol. Chem.* **159**, 97.
Halliday, N. (1938). *J. Nutrit.* **16**, 285.
Jowett, M. & Quastel, J. H. (1935). *Biochem. J.* **29**, 2143.
McHenry, E. W. & Gavin, G. (1938). *J. biol. Chem.* **125**, 653.
McHenry, E. W. & Gavin, G. (1941). *J. biol. Chem.* **138**, 471.
Quackenbush, F. W. & Steenbock, H. (1942). *J. Nutrit.* **24**, 393.
Quackenbush, F. W., Steenbock, H. & Platz, B. R. (1942). *J. biol. Chem.* **145**, 163.
Sackett, G. E. (1925). *J. biol. Chem.* **64**, 203.
Sperry W. M. & Brand, F. C. (1943). *J. biol. Chem.* **150**, 315.
Stetten, DeW. & Boxer, G. E. (1944). *J. biol. Chem.* **155**, 231.
Sure, B. & Easterling, L. (1949). *J. Nutrit.* **39**, 393.

Studies on the Analysis of Vitamins D

4. STUDIES ON THE IRRADIATION OF ERGOSTEROL AND 7-DEHYDROCHOLESTEROL AND THE ANALYSIS OF THE PRODUCTS FOR CALCIFEROL, VITAMIN D_3, AND COMPONENT STEROLS

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The chemical analysis of vitamins D in synthetic irradiation products is of considerable importance and offers more promise of success than attempts on fish-liver oils. There are, however, a number of difficulties to be met which have rendered most chemical methods of analysis unreliable. A review of published methods of analysis has been made and the difficulties discussed in earlier papers (Parts 1, 2 and 3; Green, 1951*a*, *b*, *c*).

In the experimental work to be described, analytical methods for the determination of vitamins D and related compounds have been evolved, with consideration of three factors. First, although it is realized that the irradiation products are of widely varying composition (depending mainly on the extent of photochemical reaction), the influence of this fact on the problems of chemical analysis has usually been neglected. Thus, many methods, based on examination of highly standardized types of industrial product fail in the hands of workers investigating materials of varying composition. Adequate study of the composition of the irradiation products is a prerequisite of success in vitamin D determination. Secondly, biological assay control must be rigorously applied at all stages of evolution of a chemical method for vitamin D. Thirdly, agreement between chemical and biological assays should not necessarily be accepted as evidence for quantitative determination in the absence of recovery experiments.

The ultimate means of measurement have been the iodine trichloride titration method, described by Green (1951*a*) and the antimony trichloride-acetyl chloride colorimetric method of Nield, Russell & Zimmerli (1940).

EXPERIMENTAL

Materials and apparatus

Sterols (for specification, see Green, 1951*a*). *Calciferol and vitamin* D_3. For recovery experiments only, the International Standard preparations (see note in text). *Floridin earth.* Prepared for chromatography, British Drug House Ltd. Sieved to remove particles finer than 160 mesh/in. *Antimony trichloride.* A.R. grade. *Acetyl chloride.* A.R. grade. *Chloroform.* Purified as described by Nield *et al.* (1940). *Other reagents.* (For specification, see Green, 1951 *a*, *b*, *c*.)

ICl_3 titrations were carried out on the Spekker micro-absorptiometer, using spectrum green 604 filters. $SbCl_3$-acetyl chloride colorimetric measurements were made on the same micro-absorptiometer, using spectrum blue-green 603 filters, and on the Uvispek spectrophotometer.

The $SbCl_3$-acetyl chloride determination

The directions of Nield *et al.* (1940) for the preparation of the reagent have been found satisfactory, but it has not been found advisable to keep the prepared solution up to 9 weeks, as claimed to be possible. The reagent has, in the present work, been kept up to 3 weeks, during which period it is stable. Trouble with the reagent is generally caused either by moisture absorption or by the presence of impurities in the $CHCl_3$. To avoid the former, the reagent is stored in a desiccator between experiments. The $CHCl_3$, however, is the chief cause of difficulty. Old samples develop impurities which do not appear to be easily removable, and reagents prepared with them soon deteriorate. The usual symptoms are erratic colour development and general diminution of chromogenic capacity; if these symptoms occur within 3 weeks, a new preparation of $CHCl_3$ should be obtained. With these precautions, observance of cleanliness and dryness of all apparatus in contact with the reagent, and a certain amount of practice, the reagent yields results with a degree of precision within $\pm 5\%$.

Vitamin D is determined by adding the reagent to a $CHCl_3$ solution of the test material, up to a known standard volume (in these experiments 2–5 ml.). Reagent volume should always be large compared to the volume of test solution, and never less than five times as great. This eliminates the quenching effect due to dilution of the reagent on adding it to the test solution. The addition of reagent may be carried out directly in the optical cell, if large enough, or in a stoppered vessel from which a portion for measurement may be taken. During the measurement, the optical cells should be covered with ground-glass cover slips to avoid moisture absorption, which causes cloudiness and 'streakiness' on the internal glass walls, and also volatilization, which may damage the instrument. When using the reagent in the Uvispek spectrophotometer, the instrument may be protected by incorporating a removable housing, fitting into the cell mount and designed to give maximum protection from vapours or spillage.

Providing moisture ingress into the cell is small, three consecutive readings on different solutions may be made in the same cell, which need only be rinsed with $CHCl_3$ between readings. If consecutive readings are delayed, the cells should be rinsed between readings with acetone and then blown out with air until quite dry.

$E^{1\%}_{1\,cm.}$ for the reaction with pure vitamin D_3 was determined, from a large number of readings, as 1820.

Method for the determination of vitamin D in irradiation products

The method needs no special precautions beyond those customarily observed to prevent oxidation.

Saponification. If the sample is dissolved in oil, first saponify 0·5–1·0 g. Add 0·5 N ethanolic KOH, freshly prepared with 95% (v/v) ethanol (10 ml./g. of sample) and reflux, in a 25 ml. conical flask containing a cold finger condenser, for about 5 min. on a steam bath. Wash into a separator with 10 ml. water, and extract twice with 25 ml. portions of ether. Wash the ethereal extracts successively with water, 10 ml. 0·5 N aqueous KOH, and then with water until free from alkali. Make the ether solution up to volume, after filtration if necessary.

Chromatography. Take a portion of the ethereal solution that contains preferably not more than 25 mg. of sterols and evaporate at the pump to small bulk. Add a little ethanol and blow off to dryness with an inert gas stream. Dissolve the residue in 5–10 ml. light petroleum. If the original sample was not dissolved in oil, make up directly in light petroleum. With low potency material, it may be necessary to take more than 25 mg. for chromatography. Quantities of sterols up to 100 mg. may be used, but this may cause clogging of the column and inefficient elution, unless the procedure is carried out very slowly.

The column consists of a glass tube about 15 cm. long and 1·3 cm. internal diameter, constricted at the base and fitted with a 6 cm. stem 1 mm. in bore, which ensures slow flow. The constriction is plugged with glass wool.

Activate the floridin earth by boiling for 1 min. with conc. HCl (5 g. earth to 15 ml. acid) and pour the hot stirred slurry into the tube until the column is about 8 cm. long. With practice this can be done with one tipping motion to give a perfectly even column. After the acid has passed, but without allowing the top of the column to dry, wash through with 20 ml. of 90% (v/v) ethanol in small quantities, followed by 5×5 ml. of light petroleum. During the ethanol washing, add a small filter-paper disk to the top of the column and compress the column about 5 mm. with a glass plunger. Add the sample and, after its passage, wash through with 40 ml. light petroleum. Change the receiver and elute with 100 ml. of CCl_4.

Chromatography is preferably carried out by gravity percolation and should not take less than 1 hr. or more than 2 hr. to complete. Quick passage may cause loss by non-adsorption, and prolonged passage causes some destruction of vitamin D. The rate of flow varies throughout the procedure, being slow when ethanol is being passed, but speeding up as the other solvents wet the column. Rates are adjusted by the conventional screw clip and rubber tube on the side arm of a filter flask. Suction should not be used during adsorption, but may assist during elution.

Solubility and digitonin separation. Evaporate the CCl_4 solution to dryness at the pump. To the residue, add by pipette 20 ml. of ethanol and swirl or shake in a glass-stoppered flask to dissolve. Add exactly 8·6 ml. water, with continuous swirling, to bring the ethanol concentration to 72% (v/v). Transfer immediately to a 50 ml. centrifuge tube and allow to stand 5 min. Should a precipitate form, remove by centrifuging, wash the residue with 5 ml. 72% ethanol and combine with the clear supernatant in a second centrifuge tube. Add 150 mg. digitonin dissolved, by warming, in 5 ml. 72% ethanol, stir, and allow to stand 1 hr. If no precipitate forms at the ethanol dilution stage or if only a turbidity occurs, add digitonin directly. Separate the digitonide by centrifuging, and filter the supernatant into a separating funnel. Extract twice with 15 ml. portions of CCl_4, and combine the extracts. Filter, evaporate the filtrate to dryness at the pump. Dissolve the residue in a standard volume of CCl_4.

Vitamin D measurement. The CCl_4 solution may be titrated directly with ICl_3. If the $SbCl_3$-acetyl chloride reaction is to be used, evaporate a measured portion and make up in $CHCl_3$, so that the solution contains 30–100 μg./ml. For estimation on the micro-absorptiometer, take 0·1–0·4 ml. (3–10 μg. of vitamin D) and make up to 2·0 ml. with reagent in a small test tube. Match at two levels after 3 min. against a blank cell containing reagent. Read off vitamin D content from a standard curve. For spectrophotometric estimation, rather larger quantities are necessary. Take readings (at two levels) after 3 min. at 500 mμ. against an internal blank

measurement at 550 mμ. Determine the standard 'intercept' to be subtracted, by measuring the 550–500 mμ. readings on a cell containing pure reagent. Calculate vitamin D (after subtraction of the 'intercept') from extinction values.

The whole analytical procedure is completed in about 6 hr.

Studies of the analytical method

Studies of the method were made by investigation of experimental irradiation products prepared from ergosterol and 7-dehydrocholesterol. The provitamins were irradiated in ether or benzene solution at varying concentrations and for varying periods of time, using unfiltered light from a Hanovia S. 500 lamp. All irradiations were carried out in fused-silica flasks at 2·5 cm. from the lamp, at the boiling point of the solvent used.

Weight of sample. Results obtained by the method are independent of the weight of sample taken, over wide limits. This is an important criterion of efficient separation of vitamin D from interfering materials. As indicated later, variations of the method which are known to cause faulty separation or recovery lead to large differences being found when the sample weight is varied. Table 1 shows the agreement between chemical determinations carried out on weights of irradiation product varying between 5 and 50 mg.

Table 1. *Determinations of vitamin D on varying weights of an ergosterol irradiation product*

Wt. of sample (mg.)	Chemical analysis (10^6 i.u./g.)	Bioassay (10^6 i.u./g.)
5·6	5·5	—
9·4	5·6	5·1
20·3	5·3	—
49·7	5·0	—

Saponification effect. The saponification method described is satisfactory for material containing vitamin D. Experiments on the saponification of solutions of pure vitamin D_3 in arachis and olive oils showed that, as determined by the $SbCl_3$ reaction, recovery is quantitative. Attempts to study the saponification of these solutions by the use of the ICl_3 titration method, however, brought to light a peculiar effect, which is worthy of mention.

The non-saponifiable fractions of arachis and olive oils yield negligible amounts of liberated iodine with ICl_3. If, however, the non-saponifiable fractions contain added vitamin D, on titration with ICl_3 they liberate iodine in very large excess over the amount expected to be liberated by the added vitamin D alone. Substances are present in these fractions which only react with ICl_3 in the presence of a reactive substance such as vitamin D. Addition of vitamin D before or after the actual saponification yields similar results, and length of saponification time does not effect the phenomenon. The extent of the spurious iodine development, however, increases with the amount of added vitamin D. At levels of 10 mg./g. of oil, an apparent recovery of about 175 % is obtained by ICl_3 titration. At 5 mg./g. of oil, the apparent recovery is about 125 %.

The mechanism of this reaction, initiated by the presence of vitamin D, has not been elucidated nor have the substances responsible been identified. Although interfering with saponification studies by means of the ICl_3 reaction, they cause no difficulty in the full vitamin D analysis method since they are eliminated by the chromatographic procedure.

Chromatography. This stage is preferably carried out before the extraction and precipitation procedures. If it is carried out after the latter operations, losses of vitamin D occur and recovery experiments give poor results. Prior chemical treatment appears to render vitamin D susceptible to destruction by chromatography.

Dissolving the crude irradiation product in light petroleum preparatory to chromatography meets with some difficulty in two types of product. Ergosterol products which have been irradiated for short times and which contain over 80 % of residual ergosterol are not easily soluble, and leave the poorly soluble ergosterol as a residue. The procedure here is to filter off the unchanged ergosterol rather than attempt to get the whole product in solution. The solid is washed with small amounts of warm solvent to free it of vitamin D. On prolonged irradiation, ergosterol is rendered totally soluble by its photochemical reaction products and no difficulty is experienced with an insoluble residue.

It has been found that ergosterol irradiation products change in composition on storage. Irradiation to the point of about 45 % ergosterol destruction yields a product which is obtained, after solvent evaporation, as a crystalline or semi-crystalline solid. This type of product is totally soluble in light petroleum, but leaves an insoluble ergosterol residue on treatment with 72 % ethanol. After several days in ether or benzene solution, the irradiation product changes in physical consistency to yield a non-crystalline, resinous mass (similar in appearance to the resins obtained by prolonged irradiation). The crude product is now more soluble in 72 % ethanol, but contains a new substance, almost totally insoluble in light petroleum. This substance is gelatinous whilst in suspension, and, when separated, is a white amorphous powder. Similar changes occur in the crude irradiation product after it has been freed from solvent, but the rate of change is much slower. For the purpose of chromatography, the substance may be treated as described for residual ergosterol and filtered from the light petroleum extract containing vitamin D.

Table 2 summarizes these and other changes which have been observed to occur during the ageing of an ergosterol irradiation product over a period of 2 weeks.

Table 2. *Change in characteristics of irradiation product on ageing*

Reaction or description	Freshly prepared product	Product after 2 weeks in ether
Physical appearance	Crystalline	Resinous
Solubility in light petroleum	Totally soluble	Insoluble gelatinous residue
Total reactive double bonds by ICl_3 titration	2·95	2·6
Vitamin D analysis (i.u./g.):		
(*a*) by ICl_3	$5{\cdot}8 \times 10^6$	$6{\cdot}9 \times 10^6$
(*b*) by $SbCl_3$	$5{\cdot}3 \times 10^6$	$6{\cdot}3 \times 10^6$

The product which is described in Table 2, when titrated directly with ICl_3, was found to consist almost entirely of sterols containing three reactive double bonds, thus showing an absence of appreciable amounts of lumisterol or suprasterols. During ageing, the titratable double bonds decrease. Simultaneously there is an increase (not shown in Table 2) of the total $SbCl_3$-reactive material in the crude product. In addition, there is a real increase in vitamin D content as determined chemically, which has been found in many other stored samples. Part of the change in composition is almost certainly due to a slow formation of calciferol from a precursor present in the crude product, probably the precalciferol newly isolated and described by Velluz & Amiard (1949) and Velluz, Amiard & Petit (1949). The change, as shown by these authors, has been found to occur in the absence of light. However, the formation of the petrol-insoluble substance and decrease in ICl_3 titre of the total product is due to a second simultaneous reaction, which is believed to be due to the decomposition of tachysterol. It has been found that pure tachysterol, under the same conditions, forms a closely similar petrol-insoluble decomposition product. The chemical nature of the substance has not been studied in any detail. Its ultraviolet absorption spectrum shows no bands above 230 mμ., and (unlike the petrol-insoluble suprasterol to be described later) it reacts only slightly with ICl_3.

Chromatography eliminates three types of interfering material in addition to tachysterol. The first type consists of unsaturated substances (possibly hydrocarbons or unidentified sterols) which are found in the non-saponifiable fractions of vegetable oils. These substances react with ICl_3 and, in high concentration, with the $SbCl_3$ reagent. They pass into the first light petroleum filtrate from the column.

The second substance occurs in most types of vitamin D_3 product prepared by irradiation of crude concentrates of 7-dehydrocholesterol and its elimination is important. It is reactive towards $SbCl_3$ and ICl_3 reagents and is present in considerable quantities. It behaves differently from the known sterols of the photochemical sequence on chromatographic analysis, also passing into the light petroleum filtrate.

The third substance separated by chromatography is an unidentified irradiation product of calciferol, formed also during the irradiation of ergosterol; it is described in greater detail in the section on irradiation studies.

Solubility and digitonin separation. The procedure adopted finally for the solubility separation was to dissolve the sample after chromatography in ethanol and to dilute to a concentration of 72% (v/v) with water, in order to precipitate most of the provitamins and cholesterol. Many experiments were carried out on the direct extraction of the sample with 72% ethanol, but the method was found to be unsatisfactory with some products. Although the D vitamins are preferentially extracted from irradiation products by shaking with 72% ethanol, the completeness of the extraction is affected by the weight of sample taken and also its physical consistency. Extraction from resinous products was usually good, but the crystalline products obtained during the early stages of ergosterol irradiation were difficult to extract.

It was found advisable to add digitonin in considerable excess, and extraction of the solution after digitonide separation was best carried out with carbon tetrachloride. Occasionally, during this extraction, emulsification may occur, and is due to impurities in the digitonin. Pure digitonin (not readily obtainable) never gives emulsions at this stage. If emulsification is troublesome, the preparation of digitonin used should be changed.

Considerable evidence has accumulated to indicate that the digitonin separation removes from crude irradiation products, in addition to precipitable sterols, a large amount of an $SbCl_3$-reactive sterol, which is not precipitated, but forms a soluble non-reactive digitonide, extractable from aqueous ethanol by CCl_4.

In one experiment, summarized in Table 3, 52 mg. of an ergosterol-irradiation product was, without prior chromatography, dissolved in 20 ml. ethanol

and the solution, by addition of water, brought to an ethanol concentration of 72 %. After removal of the precipitate by centrifuging (stage *A*), digitonin solution was added and the digitonides removed after 1 hr. (stage *B*). The clear filtrate was extracted into CCl_4 (stage *C*) and the extracted solution evaporated to dryness at the pump. The residue was re-extracted with CCl_4 and made up to a standard volume (stage *D*). At each of the four stages, a sample (0·5–1·0 ml.) was removed and analysed directly for total reactive material with the $SbCl_3$ reagent. The results are expressed, conveniently, in terms of 'apparent calciferol'. The test samples from stages *A*, *B* and *D* were first evaporated to dryness from their ethanolic solutions, then extracted with hot CCl_4, and after evaporation of the latter solvent, made up in $CHCl_3$ for $SbCl_3$ determination. The CCl_4 solution from stage *C* was evaporated to dryness directly, and the residue dissolved in $CHCl_3$. The complete experiment was repeated, with the exception that digitonin was not added.

Table 3. *Loss of* $SbCl_3$*-reactive sterol as soluble digitonide during vitamin D analysis*

Stage of analysis	'Apparent calciferol' ($SbCl_3$ reaction) content of original product (10^6 i.u./g.)	
	Digitonin added	Without digitonin
A After dilution	8·6	8·5
B After removal of digitonin	8·9	—
C After CCl_4 extraction	5·3	8·1
D Final extracted solution	Nil	Nil

Very little loss of calciferol occurs during the procedure, and tests on all separated precipitates showed negligible quantities of $SbCl_3$-reactive material. The addition of digitonin clearly results in the elimination of a considerable amount of $SbCl_3$-reactive sterol, as soluble digitonide; this digitonide is present at stage *B* and is broken down to its constituents by extraction with hot CCl_4. The digitonide is extracted into CCl_4 and is non-reactive, as shown by the lowered analysis at stage *C* and the absence of any $SbCl_3$-reactive material at stage *D*.

Similar results are obtained if chromatography precedes the digitonin separation, but the amount of the digitonin-removable $SbCl_3$-reactive substance separated is smaller, since a part of it is removed by the floridin column. The substance responsible is not believed to be tachysterol, which has been shown not to form such a soluble digitonide.

RESULTS

Recovery experiments

Agreement of chemical determinations with biological assays has not, without additional evidence, been accepted as a criterion during studies on the analytical method. Recovery experiments are the most critical proof that vitamin D is in fact being measured by the method. Such recovery checks have been carried out during determinations on a number of irradiation products. Some typical results are shown in Table 4. Recoveries are usually over 90 % of added vitamin D. It has been found highly important in attempting these recovery experiments with small amounts of added vitamin D to use extremely pure material. For some tests, small amounts of International Standard calciferol and vitamin D_3 were used. In others, only specimens which conformed to this high degree of purity were used. Recovery experiments with specimens of ordinary commercial purity give poor results.

Analytical studies of the irradiation of ergosterol

Irradiation method. For these studies, ergosterol was irradiated in concentrations up to 1 % (w/v) in ether and up to 12 % (w/v) in benzene. Irradiations were usually carried out on 100 ml. of solution in 250 ml. fused-silica flasks, illuminated with the Hanovia S. 500 lamp at 2·5 cm. from the flask.

The irradiation curve and comparative analyses. Ergosterol (1 g.) was irradiated in 100 ml. ether. Samples of 20 mg. were taken at intervals and assayed chemically. Additional samples at each interval were taken and, after evaporation of the solvent, dissolved in arachis oil and assayed biologically by the chick radiographic method.

Table 5 gives the results obtained by two methods of chemical determination and the bioassay figures. Fig. 1 shows the typical 'irradiation in ether' curve, where calciferol potency is plotted against irradiation time.

Agreement between the results obtained by the $SbCl_3$ determination and the bioassay is very close throughout the whole irradiation range, falling within the fiducial limits ($P = 0{\cdot}05$) of the latter assay. Results from ICl_3 titration (on the same analytical sample) are, as expected, higher than the

Table 4. *Recovery of added calciferol and vitamin* D_3 *in analysis of irradiation products*

Product type	Chemical analysis (10^6 i.u./g.)	Bioassay (10^6 i.u./g.)	Wt. of sample (mg.)	Added vitamin D (mg.)	Recovery of added vitamin D (%)
Calciferol	6·35	5·9	51·0	5·535	102
Calciferol	3·69	—	54·5	1·434	103
Vitamin D_3	1·47	1·6	43·3	2·160	102·5
	1·78	1·90	41·0	2·088	92

Table 5. *Calciferol analyses during the irradiation of ergosterol*

(1 g. irradiated)

Irradiation time (min.)	Chemical analysis (10^6 i.u./g.)		Bioassay (10^6 i.u./g.)	Limits of error of bioassay ($P = 0.05$) (%)
	ICl_3	$SbCl_3$		
0	—	<0·02	—	—
20	2·8	2·4	2·3	87–115
40	4·6	4·3	3·9	71–141
60	7·8	6·3	6·0	77–130
90	8·4	6·8	6·7	89–113
120	9·2	7·8	7·6	93–107
150	8·0	6·0	5·6	83–120
180	7·6	4·0	4·1	85–118

$SbCl_3$ figures, and these two sets of results diverge as irradiation proceeds, due to accumulation of suprasterols. Table 5 shows (and this has been confirmed on many series of analyses) that the ICl_3 figure is rarely more than 20 % higher than the true vitamin D figure as determined by $SbCl_3$ analysis, before the peak potency is reached. If irradiation is continued beyond the limits shown in the table, the ICl_3 figure remains fairly constant or falls slightly for a long period, and finally rises when suprasterol accumulation is large.

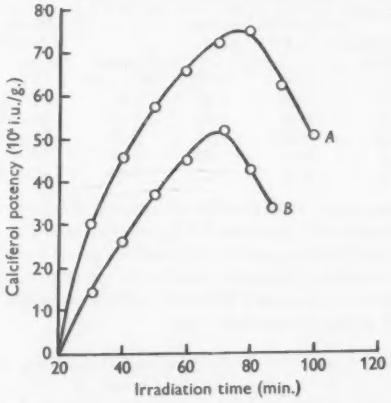


Fig. 1. Irradiation of ergosterol in ether (*A*) and benzene (*B*).

Solvent effect on irradiation. It has been recognized for many years (for example, see Bills, Honeywell & Cox, 1931) that the formation of calciferol from ergosterol takes place much faster in ether than in solvents such as benzene or alcohols. A quantitative study of the effect has been made. It was confirmed that, on irradiating similar concentrations (1 % w/v) of ergosterol in ether and benzene, calciferol formation, in the early stages of the process, was about twice as fast in the former solvent as in the latter. The typical 'irradiation in benzene' curve is shown in Fig. 1. The peak calciferol potency is also about 1·5 times to twice as great in ether as in benzene.

Ergosterol-calciferol relationships and order of reaction. During a number of experimental irradiations, the decay of ergosterol was studied kinetically, simultaneously with studies on the formation of calciferol. Residual ergosterol was determined by digitonin precipitation.

In one experiment, 5 g. of ergosterol in 100 ml. benzene were irradiated. Fig. 2 shows the ergosterol destruction and calciferol formation curves. The concentration of ergosterol does not fall off exponentially, due to the formation of a series of substances which also absorb incident light. The observed curve

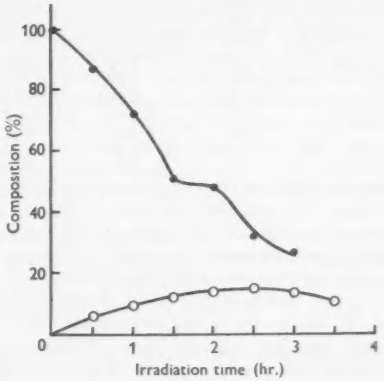


Fig. 2. Changing ergosterol-calciferol relationships during irradiation of ergosterol. ●—●, ergosterol; ○—○, calciferol.

for the rate of decay of ergosterol is quite characteristic. The initial decrease in concentration is approximately exponential, but, at about 50–60 % residual ergosterol, the curves flatten out for a considerable time during which very little ergosterol is transformed, although, at the same time, the calciferol concentration continues to increase. Finally, the ergosterol concentration falls steeply. At all initial ergosterol concentrations, and in both ether and benzene, peak calciferol potency is reached when 50–70 % transformation has occurred.

Two other factors are of importance in studies of the irradiation. The first of these is the effect of initial ergosterol concentration. During the first stages of photochemical change, using the mercury arc as the light source, it appears that calciferol is the main accumulating sterol, and, during this first period, it is useful to study its formation kinetically. It is then possible, by neglecting the intermediate reactions, to arrive at an experimental order of reaction for the ergosterol-calciferol transformation. Table 6 shows the results on a series of irradiations of ergosterol in benzene, using a wide range of initial concentrations. Irradiation was, in each experiment, stopped at 20 min. and samples analysed for calciferol. Total volumes irradiated were 100 and 200 ml.

Table 6. *Irradiation of ergosterol in varying concentrations*

(Time of irradiation = 20 min.)

Volume of solution irradiated	Concentration of ergosterol (g./100 ml.)	Potency of product (10^6 i.u./g.)	Total calciferol formed (10^6 i.u.)
100 ml.	0·25	5·68	1·42
	0·35	3·95	1·38
	0·5	3·39	1·69
	1·0	2·85	2·85
	2·0	1·93	3·86
	4·0	1·10	4·40
	6·0	0·72	4·32
200 ml.	0·25	3·18	1·59
	0·5	2·74	2·74
	1·0	1·82	3·64
	2·0	1·18	4·72
	4·0	0·62	4·92
	6·0	0·39	4·68

For a standard volume of solution irradiated in a given time, the calciferol potency of the product decreases, but not proportionally, as the ergosterol concentration increases up to a certain point. At this concentration—about 4 % for 100 ml. of solution and 2 % for 200 ml.—the order of reaction with respect to ergosterol is less than unity, and continuously varying. Above these threshold concentrations, however, the calciferol potency varies inversely with the initial ergosterol concentration, the total calciferol formed in a given time becomes constant (about $4{\cdot}5 \times 10^6$ i.u. in all volumes), and the reaction is therefore of zero order. Because the intensity of radiation is a constant in all experiments, the threshold concentration is different for each volume being irradiated; the critical factor is manifestly not concentration of ergosterol, but transparency of solution. When the volume is large enough or the concentration high enough to absorb all the incident radiation, the reaction is of zero order; the amount of calciferol formed is then independent of either volume or concentration and is a constant for a given intensity of radiation. The amount of ergosterol under these conditions is very large compared to the amount being transformed by the radiation, and its concentration remains almost constant. In later stages of the irradiation, of course, these considerations do not apply.

A second consideration of interest is the changing ratio, throughout the reaction, of the calciferol formed to ergosterol destroyed. Calciferol is formed most economically in the early stages, and for best yields based on ergosterol consumed, irradiation should not proceed beyond 15 % destruction, when about 5 % calciferol is formed. Later, ergosterol destruction becomes heavy in proportion to calciferol formation.

Analytical studies of 7-dehydrocholesterol irradiation

The irradiation of 7-dehydrocholesterol was studied by methods similar to those described for ergosterol. A 27 % concentrate of 7-dehydrocholesterol was used. In one experiment 2 g. of the concentrate dissolved in 100 ml. ether were irradiated over 5 hr., taking samples at hourly intervals. Table 7 shows vitamin D assays at each stage by chemical and biological methods.

Table 7. *Vitamin D_3 analyses on 7-dehydrocholesterol irradiation product*

Irradiation time (hr.)	Chemical analysis (10^6 i.u./g.) ICl_3	Chemical analysis (10^6 i.u./g.) $SbCl_3$	Bioassay (10^6 i.u./g.)	Limits of error of bioassay ($P = 0{\cdot}05$) (%)
1	1·26	1·33	1·32	87–115
2	2·26	2·02	2·14	83–120
3	1·89	1·73	2·05	81–118
4	1·91	1·60	1·88	80–125
5	1·88	1·46	1·56	89–112

The shape of the irradiation curve is similar to that obtained with ergosterol. Agreement between ICl_3 and $SbCl_3$ determination is usually close with vitamin D_3 irradiation products, before the peak potency is reached. Agreement between biological assays and $SbCl_3$ determinations is good.

The destruction of calciferol and vitamin D_3 by ultraviolet irradiation

A study of the photochemical destruction of the vitamins D is not only important for an understanding of the complexities of the ergosterol-calciferol relationship, but also yields useful information from the analytical aspect.

In initial experiments, 1 g. of calciferol in 100 ml. of ether or benzene was irradiated, and periodic samples analysed, by direct $SbCl_3$ determination, for residual calciferol. Some of the irradiation curves obtained are shown in Fig. 3. In addition, total reactive sterols were titrated with ICl_3, and the destruction curve obtained is shown in Fig. 4. The time ordinate on these graphs is arbitrary, as the rate of calciferol decomposition varies to a considerable extent with the age of the mercury lamp used.

If the curves obtained by direct $SbCl_3$ analysis are considered first, it is evident that destruction of calciferol in benzene is slower than in ether, but, in most experiments, the curves are similar in general shape. Destruction is rapid in the very early stages

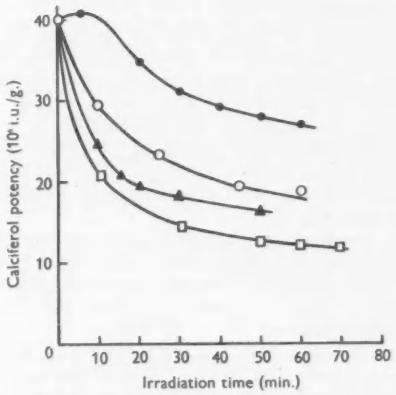


Fig. 3. Destruction of calciferol on irradiation under varying conditions. ●—●, benzene, old lamp; ○—○, ether, old lamp; ▲—▲, benzene, new lamp; □—□, ether, new lamp.

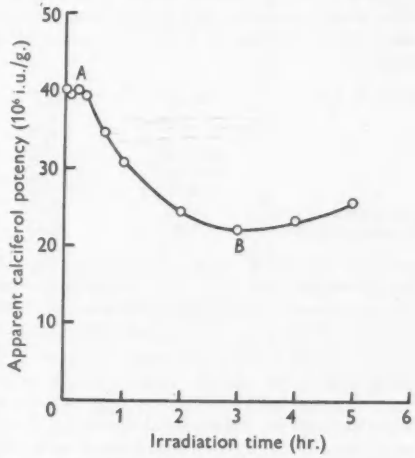


Fig. 4. Destruction of calciferol on irradiation: total reactive sterols determined by ICl_3.

of irradiation and apparently exponential. The curves then flatten out for a considerable length of irradiation time and finally descend again fairly steeply. This characteristic decomposition curve is difficult to explain.

A series of experiments was carried out, in which calciferol was irradiated in benzene at various initial concentrations. Determinations of residual calciferol were made at two intervals of 20 min. Table 8 shows that, although the total amount of calciferol destroyed in a given time increases with initial concentration, no direct proportionality exists. The reaction is of varying fractional order with respect to calciferol. Table 8 shows clearly the remarkable slowing down of destruction in the second irradiation period, becoming more marked as the initial concentration of calciferol is increased. At a concentration of 2 g./100 ml., practically no calciferol is destroyed during the second 20 min. irradiation.

Table 8. *Irradiation of calciferol in benzene*

Weight of calciferol in 100 ml. (g.)	Total calciferol destroyed (%)	
	20 min.	40 min.
0·25	52·5	76·5
0·5	45·8	51·3
1·0	35·3	39·0
2·0	26·3	26·3
1·0*	27·0	—

* 1·0 g. suprasterols added.

Attempts were made to determine whether the presence of other sterols (in particular, the accumulation of suprasterols) had a specific retarding effect on the decomposition process. Since all the products of calciferol, with the exception of the hypothetical toxisterol, are almost transparent in the range of active wavelengths, this effect, if present, would not be related to interference with light absorption. To a solution of 1 g. of calciferol in 100 ml. benzene, 1 g. of mixed suprasterols (prepared by total destructive irradiation of calciferol) was added. The solution was then irradiated and residual calciferol determined after 20 min.

As shown in Table 8, there does appear to be small specific retarding effect due to the presence of mixed suprasterols, calciferol destruction in 1 % solution being reduced from 35·3 to 27 %.

Evidence for the existence of new substances from the irradiation of vitamins D

(*a*) *Titration evidence.* If, in Fig. 4, the calciferol degradation curve as determined by ICl_3 titration is now considered, there is evidence for the existence of a number of hitherto undescribed substances produced by the photochemical destruction. During the course of the irradiation, the total titratable double bonds per molecule drops from three to a final value very close to two. The curve is not a smooth one and exhibits several points of interest. During the first few minutes of irradiation, there is a slight drop in ICl_3 titration value which is followed by a rise to the original value (point *A* in curve shown) the curve then descends to a point (*B*) where the observed double bond per molecule titration

value is about 1·5. Finally the titration value rises to about 2 and is unaffected by further irradiation. Irradiation of vitamin D_3 produces a similarly shaped curve.

At point *A* an intermediate exists which, like calciferol, has three reactive bonds, and is therefore not a known suprasterol. Spectrographic analysis of the product at this point shows only the calciferol absorption band slightly shifted to shorter wavelengths; there is no band at 248 mμ., nor has this band (due to toxisterol) ever been observed in these experiments. At *B*, there is evidence for the existence of a second intermediate which has a titre of less than two double bonds. Both these substances are photochemically labile and finally produce suprasterols.

(*b*) *Separation of new product.* In some experiments on the irradiation of pure calciferol, it was found that the crude destruction product yielded, on extraction with light petroleum, a large amount of a new insoluble substance. Suprasterols I and II, the only identified products of calciferol irradiation, are both very soluble in light petroleum. 5 g. of calciferol in 500 ml. ether were irradiated for 13 hr. in a 100 ml. fused-silica flask. After evaporation of the solvent, the resinous product was dissolved in 300 ml. light petroleum and the solution filtered through a sintered-glass filter. The washed residue was gelatinous until free from contaminant material, but dried to an amorphous, white to light-yellow powder. Yield 1·9 g.

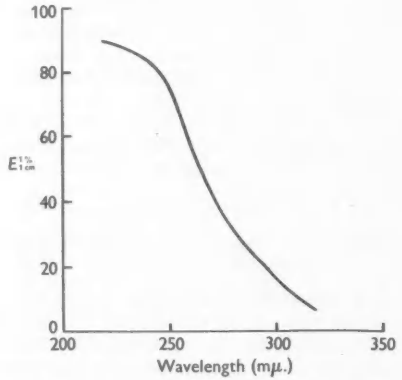


Fig. 5. Ultraviolet absorption curve of 'Suprasterol III'.

This substance, tentatively called *suprasterol III*, is soluble to the extent of about 1 % in boiling ether and is only sparingly soluble in CCl_4. Its solubility is greatly increased by admixture with crude suprasterols I and II. The substance is very soluble in ethanol and methanol and does not precipitate with digitonin. Attempts at crystallization from methanol and ethyl acetate failed. Dropwise addition of water to a methanol solution produced a gelatinous precipitation, but no crystalline material. No success met attempts to obtain a crystalline 3:5-dinitrobenzoate. Suprasterol III, dissolved in $CHCl_3$ and titrated with ICl_3, gives a figure of two double bonds. It is not affected by prolonged ultraviolet irradiation.

Formation of suprasterol III appeared to be dependent on conditions which could not be controlled. Yields were always variable and in a large number of experiments the substance was not formed. Spectrographic examination of the crude material in ethanol gave an absorption curve (Fig. 5) without bands, which is similar to that of the known suprasterols, but with greater general absorption below 260 mμ.

(*c*) *Chromatographic evidence.* If the crude product produced by total destructive irradiation of calciferol is chromatographed on floridin earth, a substance is separated which is not either residual calciferol or a known suprasterol.

In one experiment 1 g. of calciferol in 100 ml. ether was irradiated for 1 hr. After solvent evaporation, the product was dissolved in 100 ml. light petroleum and insoluble materials separated. A portion was analysed for residual calciferol by direct $SbCl_3$ estimation. The light petroleum solution (5 ml.) was then chromatographed on a standard floridin column and the two determinations were repeated on the CCl_4 eluate. The results are recorded in Table 9.

Table 9. *Chromatographic analysis of calciferol irradiation product*

	Residual calciferol (mg./g.)	Total suprasterols (by ICl_3) (mg./g.)
Before chromatography	395	618
After chromatography	385	125

Residual calciferol after irradiation remains practically unchanged after chromatography. The ICl_3 titration results are expressed in terms of 'total suprasterols' in mg./g. of original total product. The figure before chromatography is obtained by subtracting from the total titration value the contribution due to residual calciferol and then calculating from the known two double-bond titration value of the suprasterols. The figure after chromatography is obtained in the same manner and shows that a large amount of material is removed by chromatography. As already shown by Green (1951*b*), the suprasterols I and II are eluted from floridin with CCl_4, and therefore the loss is due to an unidentified irradiation product of calciferol which is not either of the former suprasterols.

Closely similar results have been obtained with products isolated from the irradiation of pure vitamin D_3.

Comparison of chemical and biological analysis on a number of irradiation products

A large number of vitamin D_3 irradiation products have been assayed chemically and biologically over a period of about 3 years, and are recorded in Table 10. Most of these were commercial products (of unknown history) prepared by dissolving the crude synthetic concentrates in arachis or cod liver oils, and ranged in potency from 0·5 to $2·5 \times 10^6$ i.u./g. Two samples were oil dilutions of much lower potency. The lowest potency material recorded in the table is about 10,000 i.u./g., but the method will deal satisfactorily with potencies down to 100 i.u. /g. The latter order of potency is, however, not usually encountered in the absence of vitamin A.

Table 10. *Comparison of chemical and biological analyses on a number of vitamins D_3 irradiation products in oil solution*

Sample number	Chemical analysis (10^6 i.u./g.)	Bioassay (10^6 i.u./g.)	Limits of error of biosassay ($P = 0·05$) (%)
235	1·78	1·70	85–117
247	2·16	2·46	81–124
233	1·26	1·56	81–124
256	0·97	0·91	89–113
272	2·48	2·99	86–116
267	2·14	2·40	87–115
297	1·17	1·34	77–130
302	1·38	1·48	78–128
316	0·51	0·54	82–122
334	0·85	0·86	87–115
374	1·88	1·80	85–117
381	1·10	1·32	90–111
384	1·24	1·01	—
385	0·65	0·76	—
386	0·99	1·10	—
392	1·46	1·60	—
403	0·88	0·86	87–115
P. 1	1·51	1·46	82–124
P. 2	1·26	1·24	83–120
S.D. 1	49,500	48,200	85–117
S.D. 2	11,200	10,800	82–122

Dr M. D. Wright, to whom the author is indebted for the large number of biological assays required during the course of this work, has supplied the following note. 'Vitamin D_3 assays are carried out in these laboratories according to the method of Olsson (1936*a*, *b*). The tarso-metatarsal distances in the chick hock joints are measured radiographically after 4–5 weeks, using diets incorporating graded doses of vitamin D_3.'

Biological assays have been carried out against the new international standard of vitamin D_3 since its adoption. Before that date, the British Standards Institution standard preparation was used, in frequent comparison with a sample of pure crystalline vitamin D_3 (which several biological tests have shown to be equal in potency to the new international standard). The B.S.I. standard preparation was shown to have 80 % of the vitamin D_3 potency of both the pure specimen and the new international standard. All the assays recorded have been brought into line and expressed in terms of vitamin D_3 standards of equivalent potency.

A scheme for full analysis of component sterols in ergosterol irradiation products

As a result of work described above and in preceding parts, a scheme suggests itself for the full analysis of the component sterols of the irradiation mixture. The sterols comprising the crude irradiation product of ergosterol can be divided into three classes (1) sterols precipitated with digitonin (ergosterol), (2) sterols reactive to ICl_3 and to the $SbCl_3$-acetyl chloride reagent (calciferol, tachysterol, pre-calciferol, and possibly an unidentified component), (3) sterols reactive to ICl_3 only (lumisterol, suprasterols and perhaps unidentified components).

The analytical scheme is shown in outline in Table 11. Ergosterol is first estimated by gravimetric separation of its digitonide. Direct estimation with the $SbCl_3$ reagent measures calciferol, tachysterol, pre-calciferol, etc. The complete analysis for calciferol is now carried out; $SbCl_3$ determination measures calciferol, and ICl_3 titration measures, in addition, lumisterol and suprasterols I and II. The latter three substances are thus obtained by difference. Tachysterol and pre-calciferol are obtained by difference from the two $SbCl_3$ determinations.

A further sample is now taken and extracted with hot light petroleum by frequent washing. The residue is determined as 'petrol-insoluble fraction', including perhaps the suprasterol III and tachysterol oxidation compound already described. The remainder of this sample, after solution in aqueous ethanol, is precipitated with digitonin to remove ergosterol, and the remainder is dissolved in dioxan and refluxed with maleic anhydride for 2 hr.

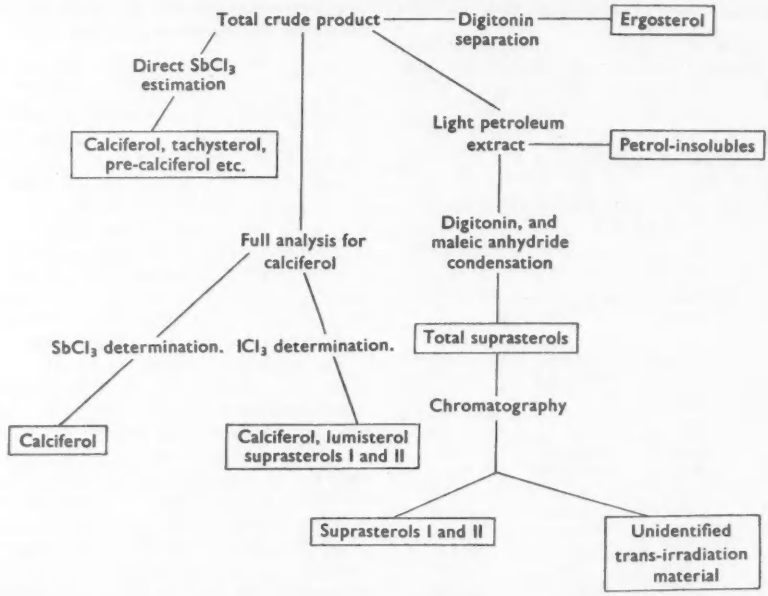
This step removes all other sterols except the total suprasterols (Green, 1951*c*), which are determined by ICl_3 titration. The suprasterol fraction may now be chromatographed on floridin, taking a CCl_4 eluate. ICl_3 titration now gives suprasterols I and II, the difference being due to unidentified over-irradiation products. Lumisterol can now be calculated by difference.

It is recognized that such a scheme as this can only be tentative and an approximation at present. The reactions on which it is based, however, are all clearly defined and the outline appears to rest on a logical basis.

DISCUSSION

In the analytical method for vitamins D which has been described, an attempt has been made to overcome the main difficulties present in the analysis of complex irradiation products. The two stages of separation used have been shown to eliminate all interfering material, with over 90 % recovery of vitamin D. The $SbCl_3$-acetyl chloride colorimetric

Table 11. *Schematic outline of full analysis of ergosterol irradiation product*



method of final measurement gives a true vitamin D assay. However, the degree of separation is such that ICl_3 titration of the final test solution yields results usually not more than 20% higher than $SbCl_3$-acetyl chloride results on samples of product which have not been over-irradiated. In most industrial types of vitamin D_3 irradiation product, it has been found that ICl_3 and $SbCl_3$-acetyl chloride results may agree to within 10%.

One of the most critical tests of the method is felt to be the close agreement between chemical and biological assays on a range of widely varying experimental irradiation products (prepared by step-wise irradiation of the provitamins, and varying in provitamin content between 10 and 100%).

Of the twenty-one samples of industrial irradiation product analysed and recorded in Table 10, in only three cases does the chemical assay fall outside the limits of error ($P = 0{\cdot}05$) of the bioassay (limits of error unavailable for four samples).

Analytical study of the irradiation process has produced several interesting results. It appears certain that the crude irradiation product is more complex than has been hitherto inferred and contains a number of unidentified substances. Precalciferol has been detected by the analytical behaviour of ageing products; whether any of the other substances indicated play a part in the direct photochemical sequence or whether they are formed in subsidiary reactions remains to be seen.

With the aid of the ICl_3 and $SbCl_3$-acetyl chloride reagents it is possible to evolve a schematic procedure for the determination of most of the individual sterols. A great deal of additional information on the composition of sterol irradiation products can probably be obtained by studies made with these two reagents.

SUMMARY

1. A method is given for the chemical determination of vitamins D in irradiation products of ergosterol and 7-dehydrocholesterol.

2. Results on a range of experimental products are in close agreement with biological assay figures.

3. Chemical and biological analyses for vitamin D_3 on twenty-one commercial irradiation products are recorded and show good agreement.

4. A study of the ultraviolet irradiation of ergosterol, 7-dehydrocholesterol, calciferol and vitamin D_3 has been made. Conditions are given for maximum calciferol formation, and factors influencing the process have been investigated.

5. The existence of unidentified substances occurring in the irradiation product has been indicated and, in some experiments, a new substance, suprasterol III, isolated.

6. A scheme is suggested for the total analysis of the components of an irradiation product.

The author wishes to thank Mr D. J. Outred and Mr D. O. Singleton for valuable assistance with the experimental work.

REFERENCES

Bills, C. E., Honeywell, E. M. & Cox, W. M. (1931). *J. biol. Chem.* **92**, 601.
Green, J. (1951*a*). *Biochem. J.* **49**, 36.
Green, J. (1951*b*). *Biochem. J.* **49**, 45.
Green, J. (1951*c*). *Biochem. J.* **49**, 54.
Nield, C. N., Russell, W. C. & Zimmerli, A. (1940). *J. biol. Chem.* **136**, 73.
Olsson, N. (1936*a*). *Arch. Geflügelk.* **10**, 11.
Olsson, N. (1936*b*). *K. fysiogr. Sällsk. Lund. Förh.* **9**, 1.
Velluz, L. & Amiard, G. (1949). *C.R. Acad. Sci., Paris*, **228**, 692.
Velluz, L. Amiard, G. & Petit, A. (1949). *Bull. Soc. chim., Paris*, **16**, 501.

Studies on the Analysis of Vitamins D

5. THE CHEMICAL DETERMINATION OF VITAMIN D IN FISH-LIVER OILS AND OTHER MATERIALS CONTAINING VITAMIN A

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The quantitative elimination of vitamin A is the most serious of all difficulties that have to be overcome in chemical methods for the analysis of vitamin D, and, up to the present, it is not generally considered that the vitamin D content of fish-liver oils can be determined by such methods with any useful degree of accuracy. In preceding papers (Green, 1951*a*,*b*,*c*) the principal methods that have been proposed by various workers on the subject have been discussed.

It has already been shown (Green, 1951*a*), that it is possible to separate vitamin A from vitamin D quantitatively by chromatographic analysis on acid-washed floridin earth. Under suitable conditions, a single liquid chromatogram can be used to free fish-liver oils from their vitamin A content without adversely affecting the vitamin D.

The present paper deals with the application of these methods to the determination of vitamin D in materials containing vitamin A.

EXPERIMENTAL AND RESULTS

Analytical method for fish liver oils

The method to be described is, with a few modifications, similar to that already described (Green, 1951*d*) for the analysis of irradiation products of ergosterol and 7-dehydrocholesterol.

Reagents. For the analysis of materials with a vitamin D potency of over 500 i.u./g., the reagents used conform to the specifications given in previous parts. Oils of lower potency (such as cod liver oils) need special precautions. The amounts of vitamin D to be measured in these oils are so small that three of the reagents (ethanol, light petroleum and digitonin) need additional purification to avoid accumulation of spurious reactive material in the final matching solutions.

Ethanol. Distilled in an all-glass apparatus over a mixture of $AgNO_3$ and NaOH. This solvent should be used for all purposes during the analysis, particularly (in 95% (v/v) concentration) for the preparation of the ethanolic KOH for saponification.

Light petroleum. Distilled from a mixture of $AgNO_3$ and NaOH and a little water. Dried over P_2O_5 and redistilled.

Digitonin. Purified from the commercial product by the method of Windaus (1925).

Weight of sample. Not less than 0·5 g. should be taken for saponification. For low potency oils, up to 5 g. may be taken.

Saponification. It may be necessary to saponify for lengths of time up to 15 min., depending on the nature of the material to be tested. Incomplete saponification may cause loss of vitamin D during chromatography.

Chromatography. This stage is as already described (Green, 1951*d*) for irradiation products. Wherever possible, no more than 20,000 i.u. of vitamin A should be passed through the column and, to this end, a suitable portion after saponification should be taken. Provided enough vitamin D is present for adequate final estimation, the amount of vitamin A passed should be as small as possible. Under these conditions, CCl_4 eluates are quite colourless; pale-yellow eluates signify over-saturation of the column with vitamin A (and possibly other substances) or incomplete washing out of the 90% ethanol used for pretreatment. With concentrates very high in vitamin A content and low in vitamin D, it may be necessary to pass as much as 200,000 i.u. of vitamin A through the column. Even using these large amounts, chromatography is usually satisfactory and eluates are almost colourless to pale yellow.

Solubility and digitonin separation. The procedure is modified for vitamin A-containing materials. After elution, the total CCl_4 eluate is evaporated to dryness at the pump in a flask fitted with a ground-glass joint. To the residue is then added 25 ml. of 72% (v/v) ethanol, and the flask stoppered and shaken vigorously for 10 min. The solution is filtered through a Whatman no. 5 paper into a 50 ml. centrifuge tube and a solution of 50–100 mg. digitonin in

72 % (v/v) ethanol added. The procedure is then as previously described.

Final estimation. The total final CCl_4 solution, or a suitable portion thereof, is evaporated to dryness and the $SbCl_3$-acetyl chloride reagent added directly to the dry residue. This procedure gives better results than if the test material is made up in $CHCl_3$ before addition of the reagent. If the latter method is used, turbidity is sometimes produced on adding the reagent. Even using the recommended method, trouble may be caused by slight turbidity in the reaction mixture; the latter must be quite clear and should be examined in a bright light before matching. Addition of a little acetyl chloride often produces clarification; alternatively, at least 10 ml. of the reaction mixture should be filtered rapidly through a small (5 cm. diameter) Whatman no. 1 filter paper previously moistened with acetyl chloride, and the last 5 ml. taken for measurement.

Extinction values were measured after exactly 3 min., on the Uvispek spectrophotometer at 500 mμ., using the 500–550 mμ. internal blank method of Nield, Russell & Zimmerli (1940), to eliminate residual interfering colours which have a small but almost constant absorption over this wavelength range.

Recovery experiments

Recovery experiments with pure vitamin D_3 have been carried out on fish-liver oils having a wide range of potencies. With low potency oils, especially, it is necessary to add extremely small amounts of vitamin D_3, and the material used must be of high purity. Good recoveries have been obtained on additions of as little as 25 μg. of vitamin D_3, using the International Standard material or a specimen of proved equivalent purity. Such commercial samples of vitamin D_3 as are available in this country are usually not suitable for use in recovery experiments.

For these experiments, a small quantity of the pure vitamin D_3 was dissolved in light petroleum at a concentration of not less than 250 μg./ml. A portion of this solution was added after saponification of the oil. The solution was always analysed colorimetrically for vitamin D_3 when the final measurement on the test substance was carried out. It is essential that the solution of vitamin D_3 should remain quite stable over the period of several hours necessary for the analysis of the oil. Specimens of vitamin D_3 which are not stable in light petroleum over this period are unsuitable for recovery use.

Results on fish-liver oils

A number of fish liver oils of widely differing potency have been assayed chemically and biologically. For certain samples obtained from other laboratories (together with statements of biological potency), individual limits of error are not available, but the limits of error ($P = 0{\cdot}05$) of these assays are unlikely to be wider than 75–125 %.

Oils of high potency in vitamins A and D. Table 1 shows the results obtained on two tunny liver oils (each containing about 290,000 i.u./g. of vitamin A), and, also, the results of the recovery experiments on these oils. Samples of 100 mg. were taken for each chemical analysis. Recoveries of over 85 % of added vitamin D_3 are shown. From the replicate chemical determinations of oil 1, in Table 1, the coefficient of variation is calculated as about 16 %. This means that in an average set of replicate assays, about two-thirds of the results will probably fall in the range 84–116 % of the mean. Biological and chemical assays show reasonable agreement.

Table 1. *Vitamin D analyses on two tunny liver oils*

	Bioassay (i.u./g.)	Chemical analyses (i.u./g.)	Added vitamin D_3 (μg.)	Added D_3 recovered (%)
Oil 1	19,800	24,200	—	—
		18,600	—	—
		19,800	475	86·1
		29,000	—	—
		25,600	251	88·6
		Mean **23,400**	—	—
Oil 2	20,000	26,400	—	—
		24,800	310	92

Medium and low potency oils. Table 2 shows results on a halibut oil and a number of cod liver oils. Halibut liver oil (500 mg.) and 5 g. of each of the cod liver oils were taken for analysis. Recovery on these

Table 2. *Vitamin D analyses on halibut and cod liver oils*

Oil used	Bioassay (i.u./g.)	Limits of error of bioassay ($P = 0{\cdot}05$) (%)	Chemical analysis (i.u./g.)	Added vitamin D_3 (μg.)	Added D_3 recovered (%)
Halibut liver oil	1950		2400	82	86
			2160	94	95
			1780	—	—
			2300	—	—
			Mean **2,160**	—	—
Cod liver oil 1	160	86–116	180	23·1	104
2	80	81–124	125	—	—
3	148	85–117	174	28·2	96
4	94	82–122	117	—	—

oils was generally very good, and chemical and biological assays were in reasonable agreement. From the four chemical determinations of the halibut oil shown in Table 2, the coefficient of variation is calculated as about 12%.

Further modifications of the chromatographic separation

The use of longer columns. Separation of vitamin D from interfering substances can be enhanced by using longer columns. Columns up to 15 cm. in length have been used with success, and vitamin D_3 recovery from these columns is good. Table 3 lists

Table 3. *Effect of longer columns during vitamin D analysis of fish-liver oils*

Oil used	Bioassay (i.u./g.)	Limits of error of bioassay ($P=0{\cdot}05$) (%)	Chemical analysis (i.u./g.) 7·5 cm. column	Chemical analysis (i.u./g.) 15 cm. column
Halibut oil	2100	—	2680	2200
Cod liver oil	150	79–127	210	180
Cod liver oil	114	85–117	160	135

a series of chemical analyses on some fish liver oils, comparing the effect of the normal 7·5 cm. with that of 15 cm. columns. Too few experiments have so far been made with these longer columns to decide as to their routine use, but they may give more promising results than columns of normal length.

Double chromatography. The effect of including a second chromatographic separation on floridin has been investigated. The usual method of analysis was followed until the final CCl_4 extract from 72% ethanol was obtained, and then, after evaporation of the CCl_4, the residue was dissolved in light petroleum and the chromatography was repeated. Providing great care was taken during the analysis, it was possible to recover 90–95% of pure vitamin D_3 from this treatment. However, in recovery experiments on fish-liver oils recoveries were only about 70%. Further work is necessary to decide whether the inclusion of a second chromatogram is warranted for routine use. The method is, however, effective in removing a great deal of residual interference and has been found particularly useful in attempts to determine the vitamin D content of high-potency vitamin A concentrates which are very low in vitamin D. These concentrates are difficult to analyse even by biological methods because of the high doses of vitamin A involved. Table 4 shows analyses of a high-potency fish-liver oil, a 'vitamin A concentrate', and a 'marine oil concentrate' of commercial origin. The vitamin A concentrate contained 227,000 i.u./g. of vitamin A and approximately 250 i.u./g. of vitamin D, and was assayed by the method described including a second chromatogram. The marine oil concentrate, a yellow semi-solid substance, contained 126,000 i.u./g. of vitamin A and about 100 i.u./g. of vitamin D. For its analysis, 2 g. were saponified and yielded about 1·2 g. of non-saponifiable matter. Because of the difficulties on putting this amount of material on the column, the non-saponifiable portion was taken up in methanol,

Table 4. *Analysis of very high potency vitamin A materials for vitamin D by a method including double chromatography*

Oil used	Bioassay (i.u./g.)	Chemical analysis (i.u./g.)
Tunny liver oil	19,500	14,500
Vitamin A concentrate	Approx. 250	213
Marine oil concentrate	Approx. 100	165

cooled to $-15°$, and the solution filtered through sintered glass at the same temperature. After evaporation of the filtrate, the analysis was continued, with a second chromatogram. As the results show, the method makes it possible to eliminate even the very high amounts of vitamin A used here, and gives results in reasonable agreement with the bioassays.

SUMMARY

1. The analysis of vitamin D in vitamin A-containing oils and concentrates by the method described by Green (1951*d*) yields results in reasonable agreement with biological assays. Recovery experiments show that, whilst total elimination of vitamin A is obtainable, very little loss of vitamin D occurs. A small amount of residual interference with the final antimony trichloride-acetyl chloride estimation remains, but is largely eliminated by the 'internal blank' method of measurement.

2. The method is applicable to fish-liver oils of a wide range of potency, but oils containing about 100 i.u. of vitamin D/g. fall near the lower limit of applicability. The detailed steps necessary for the analysis of a vitamin A-containing oil have generally to be decided for each particular sample. Thus factors such as the vitamin A : vitamin D ratio and percentage of non-saponifiable matter have to be considered.

3. When the ratio of vitamin A to vitamin D is very high, it is always necessary to use a second chromatogram, but, with normal fish-liver oils, the use of this second chromatographic separation requires caution and is not recommended. The use of 15 cm. floridin columns has been investigated and appears to be quite safe. Good results have been obtained with such columns, and their adoption may be advantageous in many analyses.

The author wishes to thank Mr D. J. Outred and Mr D. O. Singleton for their assistance with the experimental work, and Crookes Laboratories Ltd., London, for providing samples of fish-liver oils for analytical purposes.

REFERENCES

Green, J. (1951*a*). *Biochem. J.* **49**, 36.
Green, J. (1951*b*). *Biochem. J.* **49**, 45.
Green, J. (1951*c*). *Biochem. J.* **49**, 54.
Green, J. (1951*d*). *Biochem. J.* **49**, 232.
Nield, C. N., Russell, W. C. & Zimmerli, A. (1940). *J. biol. Chem.* **136**, 73.
Windaus, A. (1925). *Hoppe-Seyl Z.* **150**, 205.

Spectrophotometric Assay of Ascorbic Acid in Pure Solutions, Blood Plasma and Urine with *peri*Naphthindanetrione Hydrate

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Chemical methods of determining ascorbic acid have replaced almost entirely the earlier biological methods. Most chemical methods are based on the reducing capacity of ascorbic acid. Titration with iodine has proved to be inadequate for the determination of ascorbic acid in natural products, since they contain other reducing substances and since the colour of such products interferes with the determination of the end point of titration. Oxidation of ascorbic acid with the dye 2:6-dichlorophenolindophenol originally suggested by Tillmans (1927), has found extensive use, but in some materials the presence of reducing substances, such as 'reductones' and 'reductic acid' interferes. Several procedures have been devised to minimize these interferences. The specificity of such methods for determination of ascorbic acid is, however, not well established (Snow & Zilva, 1944). Visual titration methods by this dye are not applicable to the study of products containing water or acid-soluble pigments or reducing agents other than ascorbic acid which react with the dye at a rate sufficiently rapid to influence the end point of titration. Photometric methods (Bessey, 1938; Nochberg, Melnick & Oser, 1943) based on the 2:6-dichlorophenolindophenol reagent avoid certain of the limitations of the visual titration techniques. The fact that this dye is readily soluble in xylene makes it possible to use a xylene extraction technique (Pepkwitz, 1943) to eliminate interference from acid-soluble pigments and turbid solutions.

Reaction of derivatives of ascorbic acid with 2:4-dinitrophenylhydrazine permits the determination of ascorbic acid by methods not based upon oxidation-reduction properties. A technique described by Roe & Oesterling (1944) for total and dehydroascorbic acid, appears to avoid interference from certain types of reducing materials. Although osazones result from the reaction of sugars with 2:4-dinitrophenylhydrazine, their properties and rates of formation are sufficiently different from those of ascorbic acid derivatives to prevent their interference with the estimation. This method does not prevent interference from 'reductones' and 'reductic acid', but a means of correcting the results due to these substances has been reported (Penney & Zilva, 1945).

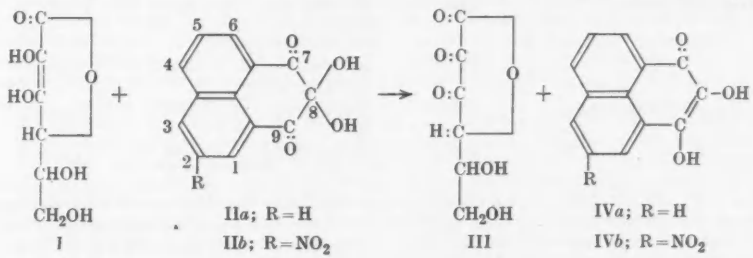
In the present work the authors propose a new spectrophotometric method for the estimation of ascorbic acid in pure solutions, blood plasma and urine. This method is based on the colour reaction obtained by the action of *peri*naphthindanetrione hydrate (II*a*) and its derivative 2-nitro*peri*naphthindanetrione hydrate (II*b*) with ascorbic acid. Preliminary spectroscopic examination of these two reagents has been carried out. Further study in this field is in progress, and details will be published later.

Nature of the colour reaction. Moubasher (1948) and Moubasher & Sina (1950) found that when solutions of *peri*naphthindanetrione hydrate (II*a*) (cf. Errera, 1913, 1914) or 2-nitro*peri*naphthindanetrione hydrate (II*b*) (cf. Calderaro, 1913) and ascorbic acid (I), are mixed at room temperature, a deep red colour rapidly develops due to the formation of dihydroxy*peri*naphthindenone (IV*a*) or dihydroxy-2-nitro*peri*naphthindenone (IV*b*) respectively. In higher concentration these products can be isolated in silky red crystalline needles, which may be identified by their melting points and mixed melting point determinations with authentic samples. The red compounds are similar to those prepared by Errera and Calderaro.

The reagents (II*a*) and (II*b*) are not only easily prepared, inexpensive and stable, but also have the following advantages:

(*a*) *Stability of the colour.* The characteristic red colour obtained is stable for about 24 hr.

(*b*) *Specificity of the reagent.* When solutions of *peri*-naphthindanetrione hydrate or its 2-nitro derivative were added to solutions of some possible interfering substances in plant, animal and biological media, under the same experimental conditions used in the case of ascorbic acid (that is to say at room temperature) no colour was developed. The following substances were tested: glucose, fructose, alanine, leucine, isoleucine, phenylalanine, lactic acid, acetoacetic acid, pyruvic acid, urea, uric acid, acetone and dehydroascorbic acid. On the other hand, reductones, reductic acid (Moubasher, 1948) and cysteine react at room temperature with *peri*-naphthindanetrione hydrate or its 2-nitro derivative producing the same red colour as in the case of ascorbic acid. These substances, therefore, interfere with the estimation of ascorbic acid by our method.



METHODS AND RESULTS

Preparation and purification of per*inaphthindanetrione hydrate* (*II a*). This compound is prepared according to Errera (1913). For purification, the trione is dissolved in hot ethanol and H_2S is then allowed to pass through the solution until complete precipitation of the crystalline red dihydroxy-*peri*naphthindenone. The precipitate is filtered off, washed with a little hot ethanol, suspended in a small quantity of water and treated with hot saturated bromine water. This solution is filtered and the filtrate concentrated by boiling. If necessary, drops of bromine water are added to oxidize the small amounts of the dihydroxy derivative that may be formed during concentration. The concentrate is allowed to cool at room temperature. The almost colourless crystals obtained are repeatedly recrystallized from aqueous ethanol (1 vol. to 1 vol.). The crystals (m.p. 270°) are recovered by filtration and dried in a desiccator. The substance sublimes *in vacuo* forming intensely red crystals. It is soluble in water and in the majority of aqueous solvents, e.g. alcohols and acetic acid. It dissolves with difficulty in benzene, benzonitrile, xylene and nitrobenzene at 100°, giving red solutions. It is practically insoluble in light petroleum (b.p. 50–60°) and dissolves in conc. H_2SO_4 forming an intense violet coloration which changes to orange after some time.

*Preparation of 2-nitro*per*inaphthindanetrione hydrate* (*II b*). This compound is prepared according to the directions of Calderaro (1916). The substance (m.p. 173°) is purified by the procedure mentioned above. The principal properties of this compound are similar to those of the mother substance.

*Preparation and purification of dihydroxy*per*inaphthindenone* (*IV a*) *and its 2-nitro derivative* (*IV b*). Ascorbic acid (Merck and Co. Inc.) (0·5 g.) in 5 ml. distilled water and 0·6 g. of pulverized *peri*naphthindanetrione hydrate dissolved in 20 ml. water are heated in a boiling-water bath for 3 min. and quickly cooled. The red silky crystalline precipitate is filtered off, washed several times with cold water and then dried; m.p. 258°, yield 0·42 g. This substance proved to be dihydroxy*peri*naphthindenone by melting point and mixed melting point with an authentic sample prepared according to Errera. (Found: C, 73·6; H, 3·8. Calc. for $C_{12}H_8O_3$: C, 73·5; H, 3·7%.) This substance is purified by repeated recrystallization from ethanol.

The dihydroxy-2-nitro derivative is prepared and purified by the procedure as above from 2-nitro*peri*naphthindanetrione hydrate. Deep-red needles, m.p. 265°, are obtained.

Spectrophotometric examination of per*inaphthindanetrione hydrate, 2-nitro*per*inaphthindanetrione hydrate and their dihydroxy derivatives*

The compounds used were pure products obtained by repeated recrystallization. All the photometric measurements recorded were made on a Hilger Barfit quartz spectrograph attached to a Spekker photometer, the light source being a condensed spark between tungsten steel. The solvent used in all cases was pure ethanol. Several concentrations of the purified substances and cells of different thicknesses were used. The spectroscopic data for the compounds are shown in Table 1 and graphically represented in Figs. 1 and 2. The absorption bands of *peri*-naphthindanetrione hydrate and its 2-nitro derivative in ethanol have intensities which can be expressed as $E^{1\%}_{1\,cm.}$ at 320 mμ. = 396 and $E^{1\%}_{1\,cm.}$ at 275 mμ. = 626 for the two compounds respectively.

The dihydroxy derivatives of these two compounds have two distinct well separated maxima, one being in the ultraviolet and the other in the visible part of the spectrum.

Estimation of ascorbic acid

Ascorbic acid was estimated in pure solution, in urine and in blood plasma by three methods.

Spectrophotometric method. Ascorbic acid was estimated through spectrophotometric determination of dihydroxy-*peri*naphthindenone produced by reduction of the reagent by

Table 1. *Absorption spectra of* peri*naphthindanetrione hydrate, 2-nitro*peri*naphthindanetrione hydrate and their dihydroxy derivatives in ethanol*

Substance	Wavelength of maxima (mμ.)	$E^{1\%}_{1\,cm.}$	$E^{1\%}_{1\,cm.}$ (mean value)	ε	log ε
*peri*Naphthindanetrione hydrate	320	396, 391, 399, 396, 394, 399	396	9,029	3·95
2-Nitro*peri*naphthindanetrione hydrate	275 inflexion 335	611, 555, 671, 665	626	17,090	4·23
Dihydroxy*peri*naphthindenone	345	517, 532, 509, 533, 517, 555, 530, 515, 506	524	11,108	4·04
	475	150, 149, 151, 149, 153, 148	150	3,180	3·50
Dihydroxy-2-nitro*peri*naphthindenone	335	393, 375, 400, 393	390	10,023	4·00
	480	86, 83, 86, 85	85	2,184	3·34

ascorbic acid in pure solution, blood plasma filtrate and urine. The amount of the reduction product thus produced is estimated by the intensity of absorption at 475 mμ. (Table 1, Fig. 2); excess of the reagent, which should always be present, does not absorb at this region of the spectrum. This is not the case if estimation is carried out at 345 mμ. (Table 1, Fig. 2) and higher values are obtained. However, good results have been obtained (absorption at 345 mμ.) if just enough reagent is added to react completely with the known amount of ascorbic acid present (in pure solution).

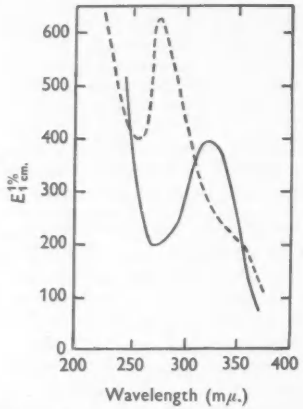


Fig. 1. Absorption spectra of *peri*naphthindanetrione hydrate, ——, and its 2-nitro derivative, – – – –, in ethanol.

Photoelectric absorption method. A standard curve of dihydroxy*peri*naphthindenone in ethanol ranging from 1 to 30 mg./100 ml. was made with different amounts of the freshly crystallized product. The percentage transmission corresponding to these amounts was recorded with reference to ethanol set at 100% transmission. A Lumetron photoelectric apparatus with a blue-green glass filter (transmitting at 475 mμ.) was used.

Roe and Kuether's method. The method of Roe & Kuether (1943) for estimation of ascorbic acid was chosen for comparison with the above-mentioned methods. A standard curve was made according to instructions of the method, and was extended to cover a range from 0·1 to 300 μg. ascorbic acid/ml.

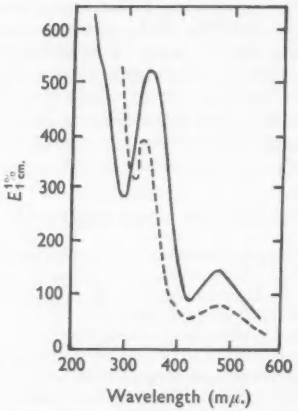


Fig. 2. Absorption spectra of dihydroxy ——, and dihydroxy-2-nitro*peri*naphthindenone – – – –, in ethanol.

Estimation of ascorbic acid in pure solution. To 5 ml. of freshly prepared standard ethanolic solution of ascorbic acid (11 mg./100 ml. ethanol) is added 1 ml. of freshly crystallized *peri*naphthindanetrione hydrate in ethanol (2 mg./ml.), mixed thoroughly and stoppered. The colour gradually develops and reaches its maximum after 10 min. The stability of the colour permits its measurement with ease any time from 10 min. to 24 hr. after the reagent has been added. The percentage transmission of the sample is recorded with reference to a blank tube of ethanol set at 100% transmission. The amount of dihydroxy*peri*naphthindenone formed is first estimated photoelectrically and then spectrophotometrically using a 5 mm. cell and the ascorbic acid present calculated from this.

Table 2 illustrates the results of ascorbic acid estimation in pure solution by the three methods.

Following the same procedure, ascorbic acid in ethanolic solution can be estimated by the derivative 2-nitro*peri*-naphthindanetrione hydrate.

Estimation of ascorbic acid in urine. The urine of subjects living on a normal mixed diet without special preparatory period was slightly acidified just after voiding (10 ml. of 5% (w/v) trichloroacetic acid to 100 ml. urine). The acidified urine was well stirred and immediately filtered through a dry filter paper. Charcoal was not used to decolorize the urine, since this leads to some loss of ascorbic acid. To 5 ml. of this clear urine was added 1 ml. of a solution of *peri*naphthindanetrione hydrate 0·5 mg./ml. in water. This amount ensures complete reaction with ascorbic acid. Ethanol was not used because it causes the precipitation of inorganic salts present in urine. The solution was then mixed thoroughly, and after 10 min. the red colour of dihydroxy*peri*naphthindenone had completely developed and was ready for estimation. The control solution prepared by mixing thoroughly 5 ml. clear urine and 1 ml. water was put at 100% transmission and the percentage transmission of the treated urine recorded. This sample was then estimated spectrophotometrically using a 2 cm. cell.

Untreated urine and water as control shows no absorption at 475 mμ. The same sample of urine was estimated simultaneously by the method of Roe & Kuether. For recovery experiments (Table 3) known amounts of ascorbic acid in water were added to urine and then estimated by the three methods. The table shows that the errors of estimation are about the same in all methods used.

Estimation of ascorbic acid in blood plasma. Blood was obtained by venepuncture from healthy individuals living on normal mixed diets. The oxalated blood was immediately centrifuged and the plasma separated. Plasma (10 ml.) was saturated with pure $(NH_4)_2SO_4$ crystals (8 g.) in a small beaker and well stirred. The mixture was then filtered with suction through a small Büchner funnel. The residue in the beaker was washed twice, with 1 ml. of 5% (w/v) metaphosphoric acid and the washings filtered. The filtrate was transferred quantitatively to a narrow finely graduated glass-stoppered measuring cylinder. About 3 ml. ethanolic solution of *peri*naphthindanetrione hydrate (0·4 mg./ml.) were added, the cylinder tightly stoppered and occasionally shaken in the course of half an hour.

Two layers separated: a lower colourless $(NH_4)_2SO_4$ layer and an upper red-coloured ethanolic layer (reduction product). The volume of the clear, coloured ethanolic layer was accurately measured and a sample portion was pipetted off and the colour estimated (ascorbic acid in this solution represents the amount of ascorbic acid in 10 ml. plasma). It has been found that there is no difference in the result if the control is pure ethanol or ethanolic extract of plasma treated exactly like the original sample. For simplicity ethanol can be used as control. Ascorbic acid in every sample of plasma was estimated by the three methods; for recovery experi-

Table 2. *Estimation of ascorbic acid in pure solution*

Ascorbic acid (mg./100 ml.)	Ascorbic acid found: Spectrophotometric method mg./100 ml.	Spectrophotometric method Error (%)	Photoelectric method mg./100 ml.	Photoelectric method Error (%)	Roe & Kuether's method mg./100 ml.	Roe & Kuether's method Error (%)
30·0	29·22	−2·6	30·55	+1·8	29·0	−3·3
24·3	23·47	−3·4	23·96	−1·4	25·0	+2·9
20·7	19·92	−3·7	19·9	−3·7	21·4	+3·3
12·0	11·9	−0·83	12·21	+1·7	12·4	+3·3
10·0	9·81	−1·9	9·96	−0·4	10·0	0·0
5·1	4·98	−2·3	5·14	+0·8	5·2	+2·0
4·0	3·9	−2·5	4·0	0·0	4·1	+2·5
2·5	2·6	+4·0	2·52	+1·0	2·6	+4·0
1·5	1·56	+4·0	1·54	+2·6	1·48	−1·3
1·0	0·95	−5·0	0·98	−2·0	0·96	−4·0

Table 3. *Estimation of ascorbic acid in urine (recovery experiments)*

Method	Ascorbic acid, mg./100 ml.: In normal urine	Amount added	Total found	Error of recovery (%)
Spectrophotometric	2·98	0·97	3·98	+2·7
	1·99	0·64	2·65	+3·2
	1·65	0·35	1·99	−2·9
	3·31	0·35	3·65	−2·8
	2·65	1·00	3·6	−5·0
Photoelectric	3·03	0·97	4·03	+2·2
	2·09	0·64	2·74	+1·5
	1·6	0·35	1·94	−2·9
	3·28	0·35	3·63	0·0
	2·7	1·0	3·67	−3·0
Roe & Kuether (1943)	3·0	0·97	4·0	+3·0
	2·0	0·64	2·66	+3·1
	1·8	0·35	2·14	−2·8
	3·5	0·35	3·84	−2·8
	2·6	1·0	3·56	−4·0

Table 4. *Estimation of ascorbic acid in normal blood plasma (recovery experiments)*

Method	Ascorbic acid, mg. /100 ml. In normal plasma	Amount added	Total found	Error of recovery (%)
Spectrophotometric	−0·79	0·5	1·28	−3·4
	−1·0	0·4	1·41	+2·5
	−1·0	1·0	1·96	−4·0
	−0·82	0·7	1·53	+1·4
	−0·82	1·2	2·18	−1·7
Photoelectric	−0·76	0·5	1·24	−4·0
	−1·03	0·4	1·43	+1·2
	−1·03	1·0	2·0	−3·0
	−0·84	0·7	1·56	+2·8
	−0·84	1·2	2·01	−2·5
Roe & Kuether (1943)	−0·72	0·5	1·2	−4·0
	−1·0	0·4	1·4	0·0
	−1·0	1·0	1·96	−4·0
	−0·88	0·7	1·6	+2·8
	−0·88	1·2	2·04	−3·3

ments (Table 4) known amounts of ascorbic acid were added to plasma which was treated in exactly the same way as normal plasma.

DISCUSSION

The advantages of the method described here are rapidity and simplicity. The reagent used is inexpensive and can easily be prepared and no special care need be taken regarding temperature or time of reaction. The possible interfering substances are reductones, reductic acid and cysteine. In Roe & Kuether's method hexoses, pentoses and glucuronic acid may interfere. Both methods seem to give about the same accuracy of result but the colorimetric method using *peri*naphthindanetrione is simpler and quicker.

SUMMARY

1. *peri*Naphthindanetrione hydrate is used as a new reagent for the spectrographic estimation of ascorbic acid in pure solutions, blood plasma and urine.

2. The 2-nitro*peri*naphthindanetrione hydrate can also be used to estimate ascorbic acid in pure solutions.

3. This reagent is easily prepared, inexpensive, specific and stable.

4. The new method when compared with that of Roe & Kuether (1943) proved to be simpler and takes less time.

REFERENCES

Bessey, O. A. (1938). *J. biol. Chem.* **126**, 771.
Calderaro, E. (1916). *Gazz. chim. ital.* **45**, no. 2, 131.
Errera, G. (1913). *Gazz. chim. ital.* **43**, 583.
Errera, G. (1914). *Gazz. chim. ital.* **44**, 18.
Moubasher, R. (1948). *J. biol. Chem.* **176**, 529.
Moubasher, R. & Sina, A. (1950). *J. chem. Soc.* p. 1422.
Nochberg, M., Melnick, D. & Oser, B. L. (1943). *Industr. Engng Chem.* (Anal. ed.), **15**, 182.
Penney, J. R. & Zilva, S. S. (1945). *Biochem. J.* **39**, 392.
Pepkwitz, L. P. (1943). *J. biol. Chem.* **151**, 405.
Roe, J. H. & C. Kuether, A. (1943). *J. biol. Chem.* **147**, 399.
Roe, J. H. & Oesterling, M. J. (1944). *J. biol. Chem.* **152**, 511.
Snow, G. A. & Zilva, S. S. (1944). *Biochem. J.* **38**, 458.
Tillmans, J. Z. (1927). *Z. Untersuch. Lebensmitt.* **54**, 33.

The Inhibition of Histaminase by Amidines

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In a study of the inhibitory action of certain amidine derivatives on the enzyme amine oxidase it was found that the inhibition depended not only on the presence of the amidine group but on the configuration of the molecule as a whole (Blaschko & Duthie, 1945*a*). This study has recently been extended to a number of *iso*thiourea derivatives (Fastier & Hawkins, 1951).

Histaminase ('diamine oxidase') is also inhibited by some of these amidines (Blaschko & Duthie, 1944), and in the present paper we have examined the inhibitory action in more detail. The results show

that the relation between chemical structure and inhibitory action is quite different for the two enzymes which catalyse the oxidative deamination of amines.

METHODS

The enzyme was obtained from pig kidney; its preparation from acetone-dried powder has already been described (Blaschko & Duthie, 1945*b*). By dialysing the extracts for a few hours against tap water the O_2 consumption of the control can be reduced without affecting the activity of the enzyme, and this is not altered in the presence of the amidines. The latter were added, together with the substrate (cadaverine), from the side arm of the manometer vessel at the start of the experiment. The flasks were set up as follows: main flask: 1·6 ml. extract; side bulb: 0·2 ml. of 5×10^{-2} M-cadaverine dihydrochloride and 0·2 ml. water or inhibitor; inner tube: 0·3 ml. N-KOH. The gas phase was O_2 and the temperature 37·5°.

The amidines examined include those studied by Blaschko & Duthie (1945*a*) and some of the *iso*thiourea derivatives tested by Fastier & Hawkins. Decamethylene diguanidine dihydrochloride—prepared from the commercially available carbonate—was tested as an inhibitor of both amine oxidase and histaminase.

The inhibitors were examined in concentrations of 10^{-3} M or less. Percentage inhibitions were calculated from the amounts of O_2 consumed during the first 30 min. of the reaction.

RESULTS

Straight-chain monoamidines:

$CH_3.(CH_2)_n.C(:NH).NH_2$ (Table 1).

The inhibitory action of the members of this group was small. Table 1 shows that there was no marked effect of chain length.

Straight-chain diamidines:

$NH_2(NH:)C.(CH_2)_n.C(:NH).NH_2$ (Table 2).

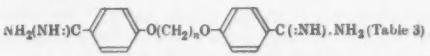
The compounds of this series were more active as inhibitors than the monoamidines. The length of the polymethylene chain was without influence on the degree of inhibition; substances with a chain length from $n = 7$ to $n = 16$ had approximately the same inhibitory action. This is in marked contrast to the results with amine oxidase (Blaschko & Duthie, 1945*a*), where the inhibitory action is at a maximum for the compound with eleven CH_2 groups.

Table 1. *Inhibition of diamine oxidase by straight-chain monoamidines*

(Type compound $CH_3(CH_2)_nC(:NH)NH_2$. Inhibitor concentration 10^{-3} M.)

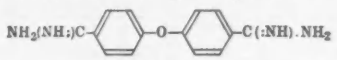
n	3	4	8	10	14
Inhibition (%)	14	6	24	16	3
	26		33		

Diamidines:



The inhibitory activity of the substances in this group was greater, and they were therefore examined in a molar concentration of 10^{-4}. The compound with one CH_2 group was most, and that with ten CH_2 groups least active as inhibitor, but the substances with two, three, four, five and six CH_2 groups all had very much the same inhibitory activity. This group includes propamidine ($n = 3$) and pentamidine ($n = 5$), both strong inhibitors of amine oxidase.

The closely related compound phenamidine,



also was a strong inhibitor of diamine oxidase, 10^{-4} M inhibiting by 90 %.

Guanidine derivatives. The two monoguanidines,

$CH_3(CH_2)_6NHC(:NH).NH_2$

and $CH_3(CH_2)_{12}NHC(:NH).NH_2$,

were too insoluble to be tested in a concentration of 10^{-3} M; in 10^{-4} M concentration the inhibitions were 4 and 5 % respectively; this is probably within the limits of experimental error.

The series of straight-chain diguanidines,

$NH_2(NH:)CHN(CH_2)_nHNC(:NH).NH_2$,

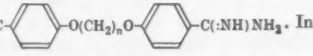
included substances with a high affinity for diamine oxidase, and the effect of chain length on inhibitory

Table 2. *Inhibition of diamine oxidase by straight-chain diamidines*

(Type compound $NH_2(NH:)C(CH_2)_nC(:NH)NH_2$. Inhibitor concentration 10^{-3} M.)

n	7	8	9	10	11	12	12	14	16
Inhibition (%)	80	73	77	92	74	57	76	76	78
				68		78	79	82	85
				84					

Table 3. *Inhibition of diamine oxidase by certain aromatic diamidines*

(Type compound  Inhibitor concentration 10^{-4} M.)

n	1	2	3	4	5	6	10
Inhibition (%)	81	70	49	61	73	63	25
	94	74	51	59	72	61	31
	95				63	72	
					75		

action was marked. Fig. 1 gives the percentage inhibitions observed with a concentration of 10^{-4} M, plotted against chain length. With the lower members of the series inhibitions were high, although slightly irregular, but beyond $n=8$ inhibitions fell off, and for the compound with $n=18$ inhibitory action was slight.

The relation between chain length and inhibition in this series was characteristically different from that found for amine oxidase. Fig. 1 also shows the percentage inhibitions observed with amine oxidase for an inhibitor concentration of 10^{-3} M; most of these data are from an earlier paper (Blaschko & Duthie, 1945*a*), but recent results with decamethylene diguanidine have been included.

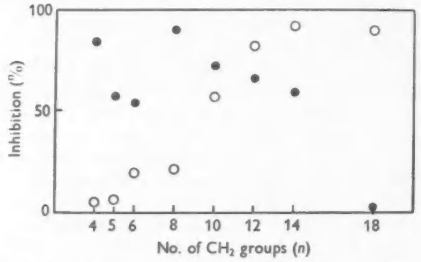


Fig. 1. Inhibition of amine oxidases by diguanidines. ●, histaminase, inhibitor concentration 10^{-4} M; ○, amine oxidase, inhibitor concentration 10^{-3} M. Each dot or circle represents the mean of several experiments.

A diguanidine derivative of spermine,

$$NH_2(NH:)CNH(CH_2)_3NH(CH_2)_4 NH(CH_2)_3NHC(:NH).NH_2,$$

gave an inhibition of 88 % in 10^{-3} M concentration.

Monoisothiourea derivatives. Ten members of the series $CH_3(CH_2)_nS.C(:NH)NH_2$ have been tested, the first nine ($n=0$–8) and the twelfth ($n=11$). In a concentration of 10^{-4} M, only the first, *S*-methyl *iso*thiourea, caused an inhibition of 25 %; on raising the concentration to 10^{-3} M, values of 52 and 69 % were obtained. None of the higher homologues affected the enzyme in a concentration of 10^{-4} M.

The first three members of the series:

$$C_6H_5\text{-}(CH_2)_nS.C(:NH)NH_2,$$

viz. *S*-benzyl, *S*-2-phenylethyl and *S*-3-phenyl-*n*-propyl *iso*thiourea, were without appreciable inhibitory activity at 10^{-4} M concentration.

Diisothiourea derivatives.

$$NH_2(NH:)C.S(CH_2)_nS.C(:NH)NH_2.$$

Eight compounds of this series were examined, the first six ($n=1$–6), the decamethylene and the dodecamethylene derivatives. Fig. 2 gives a summary of our experiments in which these substances were tested in a molar concentration of 10^{-4}. The methylene derivative ($n=1$) was found to be without inhibitory action, and *SS'*-ethylene di*iso*thiourea ($n=2$) was less active than the higher members which all inhibited the enzyme to about the same extent.

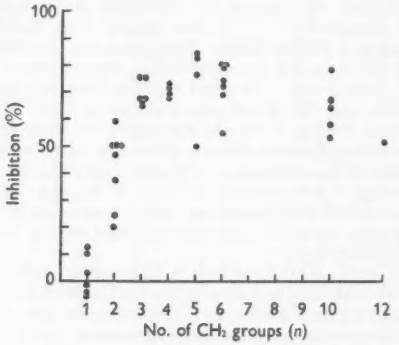


Fig. 2. Inhibition of histaminase by di*iso*thioureas. Inhibitor concentration 10^{-4} M. Each dot represents one experiment.

Stilbamidine and dimethylstilbamidine (Table 4) Stilbamidine is a very strong inhibitor of diamine oxidase, and Table 4 shows that the dimethyl

Table 4. *Inhibition of diamine oxidase by stilbamidine and dimethylstilbamidine*

Inhibitor concentration (M) ...	10^{-3}	10^{-4}	10^{-5}
Inhibition by stilbamidine (%)	100	89	50
Inhibition by dimethylstilbamidine (%)	100	87	61

derivative has very much the same affinity; even in 10^{-5} M concentration the inhibitions were still about 50 %. The percentage inhibitions differ slightly from those previously reported (Blaschko & Duthie, 1944); this is possibly due to differences in experimental conditions, since the earlier results were obtained with a fresh aqueous extract of pig kidney.

Aneurin. According to Zeller (1939), aneurin is an inhibitor of diamine oxidase. This was confirmed in our experiments, but the percentage inhibitions were rather low. With 10^{-3} M-aneurin the inhibition was 40 % and with 10^{-4} M-aneurin 5 %.

DISCUSSION

Guanidine was the first amidine compound which was found to inhibit histaminase (Blaschko, 1938). Zeller (1938) showed that the two diguanidines, arcaine ($n=4$) and Synthalin A ($n=10$) were even stronger inhibitors. The experiments reported in this paper show that it is always in the series of compounds with two amidino groups that the strong inhibitors are found; the monoamidines are less active. This also finds a parallel in the observations of King, Lourie & Yorke (1938) on the trypanocidal

action of amidine derivatives *in vitro*, the diamidines being more active than the monoamidines.

Many of the diamidines studied in this paper cause an increase in the blood histamine concentration (MacIntosh & Paton, 1949), due to the release of histamine from the tissues. Some of the compounds which were particularly active as inhibitors of histaminase in our experiments were also highly active in MacIntosh & Paton's experiments. There is no general parallelism between antihistaminase activity and histamine release, but in view of the observations here reported the possibility must be considered that some of these substances exert an antihistaminase effect also in the living animal.

SUMMARY

1. The inhibitory action of amidine derivatives on the histaminase of pig kidney has been studied.

2. Diamidines, diguanidines and di*iso*thiourea derivatives have an inhibitory action.

3. Stilbamidine and dimethylstilbamidine are strong inhibitors of the enzyme.

4. The relation between chain length and inhibition has been studied and the results are discussed.

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REFERENCES

Blaschko, H. (1938). 16*th Internat. Physiol. Kongress Zurich. Kongressbericht*, **3**, 73.
Blaschko, H. & Duthie, R. (1944). *Biochem. J.* **38**, xxv.
Blaschko, H. & Duthie, R. (1945*a*). *Biochem. J.* **39**, 347.
Blaschko, H. & Duthie, R. (1945*b*). *Biochem. J.* **39**, 478.
Fastier, F. N. & Hawkins, J. (1951). *Brit. J. Pharmacol.* **6**, 256.
King, H., Lourie, E. M. & Yorke, W. (1938). *Ann. trop. Med. Parasit.* **32**, 177.
MacIntosh, F. C. & Paton, W. D. M. (1949). *J. Physiol.* **109**, 190.
Zeller, E. A. (1938). *Helv. chim. Acta*, **21**, 1645.
Zeller, E. A. (1939). *Helv. chim. Acta*, **22**, 837.

A New Type of Preparative Electrophoresis Cell

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The electrophoresis apparatus devised by Tiselius (1937) is now available in many laboratories. It is predominantly used for analytical work, but can also be used for preparing electrophoretically pure materials in small quantities by virtue of the fact that after the migration of the various components of a mixture has taken place for a time, the fastest and slowest may be isolated by lateral displacement of the cell sections, the boundaries having been compensated to suitable positions. The disadvantages of this method are twofold. In the first place, at the conclusion of one such separation the whole apparatus must be taken out of the refrigerated bath, and at least partially dismantled in order that the desired material may be removed, and if more material is required, the apparatus must be reassembled, refilled, and replaced in the bath before another run can be started. In the second place, when removing components obtained in this way, it is difficult to avoid dilution with the buffer which fills the remainder of the cell compartment. These difficulties have to a certain extent been overcome by a semi-continuous procedure developed by Maclagan & Bunn (1947) with the insertion of capillary tubes inside the channel of the long centre section Tiselius U-tube, but the method is not entirely satisfactory to operate.

EXPERIMENTAL

Description of cell. The type of cell described here enables both the disadvantages mentioned to be overcome, while still using the standard auxiliary equipment belonging to the analytical cell. In principle it is based on the type of cell first described by Svensson (1942) and later simplified and modified by him (Svensson, 1946). Fig. 1 shows that the cell is identical with the standard Tiselius model with the long centre section, except for the fact that five capillary openings are provided, two at the top of either arm of the cell, two at the bottom, and one in the base section which joins the two arms. These openings allow material to be introduced into and withdrawn from the cell, while the apparatus as a whole remains in the refrigerated bath. In order that this should be possible, arms of capillary tubing are attached to the outlets either by rubber sleeves or by ground-glass joints, and by means of taps in these tubes the flow of liquid into and out of the cell may be controlled. The formation of protein-buffer boundaries in this cell takes place by flow of liquid, the

protein solution being introduced to the cell through the opening in the base section, rising in either arm stabilized by density difference, until it reaches the opening in the cell which is acting as an outlet. The boundary may be sharpened by techniques described below.

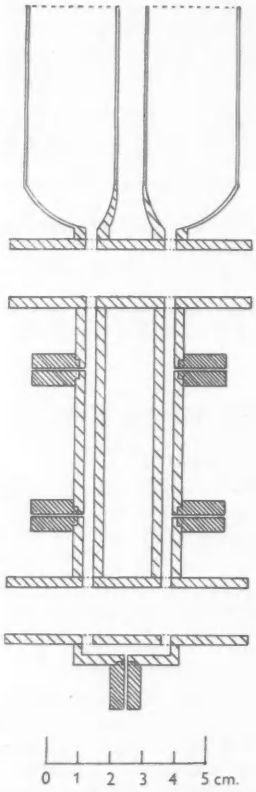

Fig. 1. Longitudinal section of the cell components.

Fig. 2 shows the cell assembled with the side arms in position. The sections are squeezed together after greasing the ground-glass surfaces as in assembling the normal cell. Since no lateral movement of the section takes place once the assembly is completed, a rather more viscous grease can be used than with the analytical cell; ordinary vaseline has proved satisfactory. This is of advantage in preserving a water-tight seal when the apparatus is subjected to mild internal hydrostatic pressure in the course of a separation. For the same reason, the downward pressure provided by the metal spring strips on top of the cell should be greater than with the analytical model. The capillary side arms, if attached by means of rubber sleeves as shown here, should be pressed firmly in place so that the flattened end of the arm is flush with the end of the projection on the cell, in order to reduce the volume of the liquid contained in the arm to a minimum. The bore of the tubing used should also be as small as possible; a bore of 1 mm. has been found satisfactory.

It will be seen that the stand in which the cell is assembled differs from the stand for the analytical cell in that the pneumatic pumps, or alternative devices by which lateral movement of the standard cell sections is effected, are replaced by metal plates which hold this cell firmly in position when it has been assembled. These plates are removable, and are adjusted by means of a bolt and wing nut projecting through a slot in the base of the stand; they also supply some support and alignment for the capillary side arms. It is with respect to this stand only that the accessories for this cell differ from those of the normal analytical model.

The electrode vessels are attached to the upper part of the cell in the usual manner (Fig. 3). The Ag/AgCl electrodes are fitted through rubber bungs so that the apparatus can be sealed, tubes being provided through which saturated KCl may be introduced to surround the electrodes. Air vents with taps are also provided in each bung to allow all the air in each vessel to be readily displaced. The two inlets to the connecting arms of the electrode vessels, through which hydrostatic equilibration occurs in the analytical apparatus, are connected by a Y-piece to a container from which buffer may be introduced into either arm of the cell at will.

Operation of cell. In use, the apparatus is first assembled and filled with the buffer in which the protein separation is to be carried out. The bulk of the filling may be done through the open top of an electrode vessel, but the final filling is best done through the capillary leading to the base of the cell, in order to remove any bubbles from the U-tube. The side arms and the air vents on the electrode vessels are then opened in turn and washed through to ensure that no air remains in the apparatus. When filling is complete, all the outlets are closed and a hydrostatic pressure of 20–30 cm. buffer imposed on the system through the capillary to the base of the cell for 2–3 min. to reveal any leaks in the rubber connexions or ground-glass surfaces. If no leaks occur, the pressure is removed and the apparatus placed in the refrigerated bath at 0·2–0·5°. One of the air vents in the top of the electrode vessels is opened to allow for contraction of the buffer. After 30–45 min. 25 ml. saturated KCl, precooled to 0·2–0·5°, is run in to surround each electrode through the tubes provided, the displaced buffer first filling the air vent that was opened to allow for thermal contraction, and later flowing out through any one of the side arms of the cell opened for the purpose.

The apparatus, now in thermal equilibrium with the bath, is again sealed.

The protein-buffer boundaries are formed for the start of the electrophoresis run by introducing the protein solution through the inflow capillary to the base of the cell, and displacing buffer through the appropriate side arm. In general, when carrying out operations with this cell, it is advisable to open the outflow point before the inflow, and to close the inflow point before the outflow; this prevents a static pressure building up in the apparatus which, because of a slight elasticity in the system, may give rise to unwanted boundary displacements when released.

A container such as a graduated pipette is attached to the inflow capillary; one of the side arms to the lower outlets is opened, and the tap in the inflow tube is gently opened until the displaced buffer emerges from the side arm at a rate of about one drop every 2 sec. When 3–4 ml. solution have run in, a very diffuse boundary rises from the bottom of the tube towards the open lower outlet. When this outlet is reached, the boundary sharpens until the diffuse region in which the protein solution and buffer are mixed is displaced. The inflow is now shut off, and the outflow side arm closed. The same process is now repeated on the other side of the cell, but the side arm to the upper outlet rather than the lower is opened, and the protein, rising from the bottom of the tube, forms a boundary at the upper level.

These two boundaries may be sharpened by allowing buffer to flow down from the pipette attached to the Y-piece into the U-tube and out through the capillary at the level of the boundary concerned. In general, boundaries formed by the flow-through method will initially be more diffuse on one side than on the other, and may be sharpened by a stream of material on the diffuse side, boundaries diffuse at the upper side being sharpened by buffer, and those diffuse at the lower side by protein solution.

In this way, boundaries are produced in the U-tube in the usual positions for the start of an electrophoresis run, one at the top of a limb, and one at the bottom of the opposite limb. Two precautions must be observed. The separated components are removed through the upper outlets at the end of the run, and it is desirable that these should be free from the protein mixture; consequently they must be washed through with buffer as a final step in boundary formation at the upper level. Also, unless the lower side arms are filled with protein solution at the beginning of a run, convections occur when the protein solution in the cell migrates above the level of the outlet. Formation of the lower boundary is thus completed by a flow of protein as a final step.

If the current is turned on, electrophoretic migration now takes place in the usual way. This is allowed to proceed until the front edge of the leading component in the ascending limb reaches the level of the upper outlet capillary. To enable this level to be observed optically, two small holes are drilled in the slotted metal diaphragm fitted in front of the cell to cut off light other than that passing through the channels of the U-tube. These holes are at the levels of the pairs of outlet capillaries, and allow a thin pencil of light to pass through the optical system at each of these levels. On the plane at which the usual electrophoretic boundary pattern appears, these light beams give rise to two horizontal streaks of light in positions corresponding to the level of each outlet capillary (Fig. 4). These act as markers, and enable the position of the migrating components relative to the outlets to be seen. A mask on the outer bath window makes it possible to cut off these rays of light if a photograph of the boundary pattern is required for analytical purposes. In the model supplied by Messrs Hilger and Watts, the light marker beam device is replaced by the following arrangement. Two lines engraved on a grey screen positioned in the plateholder, are aligned to the capillary openings, the position of the lines being set to pointers attached to the cell mount.

When the run has been completed, the electrophoretic fractions may be removed, the fastest moving components in the ascending limb and the slowest in the descending limb being displaced through the upper side arms by feeding protein solution into the base of the U-tube. To remove the leading components, for example, the tap in the upper side arm on the ascending limb is opened, and the protein inflow adjusted to give a slow displacement of liquid through this arm. The electrophoresis pattern will be seen to move towards the upper marker light, forming a new protein-buffer boundary at this level as the components flow out through the side arm (Fig. 4). This displacement is continued until the leading edge of the peak corresponding to the first undesired component reaches this marker level; the protein inflow is then turned off to stop unwanted material from entering the side arm. During this process the liquid emerging from the arm is collected; the first fraction of this, however, consists of buffer with which the arm was filled before starting the run, so that a volume of liquid equal to that of the side arm (here 1 ml.) is rejected. At the end of this displacement, the side arm contains a part of the desired material; this is obtained by allowing buffer to flow into the U-tube on the ascending side from the pipette attached to the Y-piece until the liquid in the side arm is displaced. This completes the removal of the fractions on the ascending side.

From the descending limb the slower moving components can be removed in the same way through the upper side arm. If the slowest component has travelled an appreciable distance away from the upper marker, it is unnecessary to start collecting

the displaced material until the trailing edge of the boundary has moved up to the side arm. After allowing for the volume of the arm itself as before, the protein fractions can be collected.

On account of the narrow bore of the capillary side arms there is very little mixing of buffer with separated protein components during the sampling procedure. Consequently the pure components are obtained at effectively the same concentration as that with which they occur in the protein mixture.

To continue the separation, new boundaries must be formed. In order to prevent progressive migration of the potassium chloride from the electrode vessels ultimately reaching the U-tube and making further runs impossible, alternate runs are carried out in opposite directions, with reversal of current. Consequently, the initial boundary positions must be inverted, the upper boundary being formed in the limb that was previously the ascending and vice versa. The lower boundary is formed by washing residual boundaries through the lower outlet with buffer from above, and the upper by washing out through the upper outlet with protein solution from below. They are sharpened as described above, and a second run started with reversed current.

General remarks

For the first run about 15 ml. of protein solution are generally needed to form the boundaries, and for subsequent runs about 7–10 ml. are needed in order to remove separated components and re-form the boundaries in the new positions.

It has been found when working with human plasma and plasma fractions at concentrations of 2–3 g./100 ml. in phosphate buffer, pH 8, I 0·2, that currents in the region of 20–30 mA. can be used without convections occurring. This enables at least two separations to be carried out in the course of a day, for with proteins of high mobility such as albumin and α-globulin each run is complete in 2·5–3 hr., depending on the current used. The apparatus can also be operated overnight at currents of 4–10 mA.

The normal manipulation of the apparatus may be modified in many ways. It is possible, for instance, to continue the run until the leading component has passed the level of the upper outlet, and to stop the current when the marker line comes between the first and second components. The first component may then be obtained by displacing with buffer from above, or the mixture of first and second components by displacing with protein from below. If the protein mixture being separated is not plentiful, a certain amount can be saved during the re-forming of the boundaries by letting downward displacement by buffer on one side produce an upward displacement on the other. The potentialities of the apparatus and its flexibility in various situations are realized more fully as experience is gained of it in use.

The limitations of the apparatus are those common to all in which electrophoretic migration takes place in solutions stabilized by gravity. The main disadvantage is that in a protein solution consisting of more than two components, only the fastest and the slowest of these may be obtained electrophoretically pure after a single migration. Separation of proteins with closely similar mobilities is made difficult by the limitation on the migration distance imposed by the length of limb available, and the occurrence of the stationary delta boundary. In the absence of the latter, the migration distance could be increased by a displacement of liquid in the system in the direction opposite to that of protein migration, but the delta boundary may not be displaced beyond the base of the U-tube without upsetting the density equilibrium.

This tube finds its most useful application when relatively large quantities of material are available, so that the semi-continuous separation effected can be used to full advantage.

SUMMARY

1. A modified form of electrophoresis U-tube is described, which is particularly suitable for semi-continuous preparative operation.

2. Details of the method of operating the apparatus are given.

REFERENCES

Maclagan, N. F. & Bunn, D. (1947). *Biochem. J.* **41**, 580.
Svensson, H. (1942). *Ark. Kemi. Min. Geol.* **15** B, no. 19. p.1.
Svensson, H. (1946). *Ark. Kemi. Min. Geol.* **22** A, no. 10, p.144.
Tiselius, A. (1937). *Trans. Faraday Soc.* **33**, 524.

EXPLANATION OF PLATES

PLATE 2.

Fig. 2. The cell assembled on metal stand with side arms in position.

Fig. 3. The complete U-tube assembled with electrode vessels and filling pipettes.

PLATE 3.

Fig. 4. Ascending limb photographs of a protein mixture obtained from a horse antitoxic serum. Total protein 3 g./100 ml. in phosphate buffer pH 8, I, 0·2. Migration 3·5 hr. at 20 mA. (*a*) Initial boundary. (*b*) Initial boundary and capillary level marker lights. (*c*) Electrophoretic pattern of protein mixture at completion of migration, and prior to removal of components. Peaks, left to right: albumin, α_1- and α_2-globulins, β_1- and β_2-globulins, δ-boundary. (*d*) As (*c*) but after removal of the fraction consisting of albumin, α_1- and α_2-globulins.

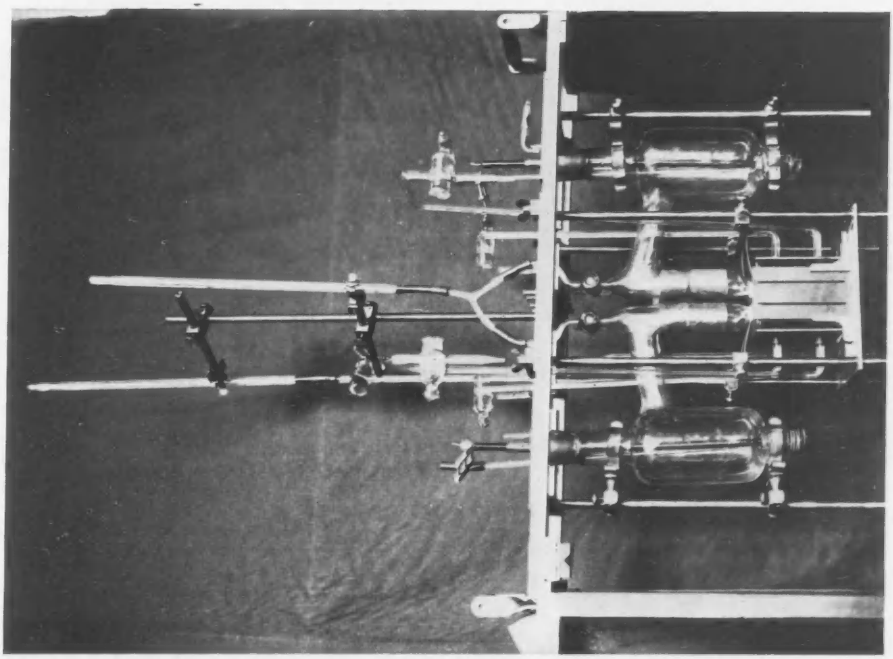

Fig. 3.

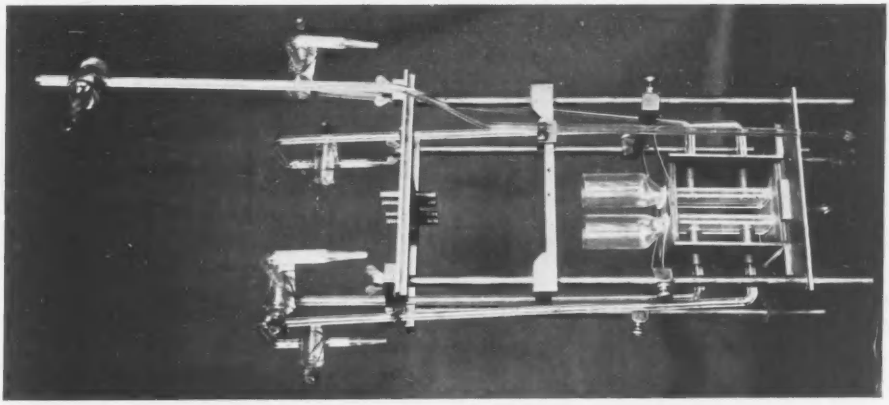

Fig. 2.

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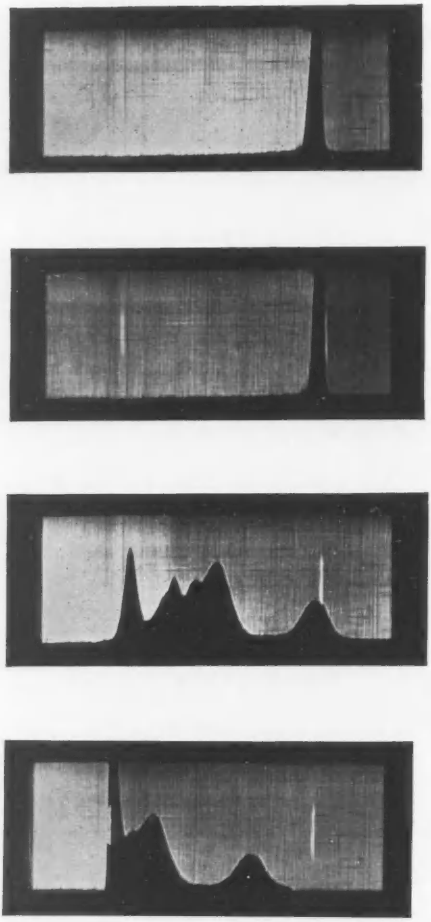

Fig. 4.